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THE EVOLUTION OF VOLTAGE-GATED SODIUM CHANNELS AS INTERPRETED FROM A STUDY OF SODIUM CURRENTS AND CHANNELS FROM THE HYDROZOAN JELLYFISH, *POLYORCHIS PENICILLATUS*

by

John David Spafford ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

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September 4th, 1998

Date submitted to the Faculty of Graduate Studies and Research

"Speculations about evolution have an irresistible fascination, partly because they are hard to prove wrong, partly because they make us think about origins and look for clues in development. Cnidarians may have been the first metazoans to evolve, although this seems rather unlikely in view of the fact that they are all carnivores, preying on other metazoans. Given, however, that of the surviving phyla they alone retain the presumably ancestral (diploblastic) body plan, it becomes especially interesting to look at their nervous systems for clues as to how nerves evolved. This is not just a selling point for use in grant applications. There really is no better group in which to look for clues."

Excerpt from the first paragraph addressed at the plenary lecture by the "grandfather of jellyfish neurobiology" George O. Mackie for the NATO Advanced Research Workshop: "Evolution of the First Nervous Systems", St. Andrews University, Scotland, July 1989.

University of Alberta

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Implications for the Evolution of Voltage-Gated Sodium Channels from a Study of Sodium Currents and Channels from the Hydrozoan Jellyfish, Polyorchis penicillatus* submitted by John David Spafford in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology and Cell Biology.

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ABSTRACT

Voltage-gated sodium channels are responsible for the rapid depolarization of membranes that leads to propagated action potentials. Sodium channels are considered to be the most recently evolved member of the voltage-gated ion channel superfamily, probably derived from Ca^{2+} channels. Sodium channels likely evolved to mediate high frequency signaling and rapid propagation to coordinate the activities of multicellular organisms, and thus may have been coincident with the appearance of the Metazoa.

Cnidarians are the simplest eumetazoans with a discrete nervous system and rapid, transient Na^+ currents. Cnidarians are considered to have split from the main lineage early in metazoan evolution and thus are uniquely suited for providing clues to the evolution of Na^+ channels.

Sodium currents in the hydromedusa, *Polyorchis penicillatus* were shown to be unusually insensitive to classical Na^+ channel drugs, like tetrodotoxin, but neither are they sensitive to Ca^{2+} channel drugs. Two electrophysiologically distinct Na^+ currents were described in swimming motor neurons and "B neurons". Nonetheless, only one full-length Na^+ channel cDNA could be found from *P. penicillatus*. The 5.8 kb cDNA has major structural features of typical Na^+ channels, especially in areas responsible for ion selectivity, voltage-sensing, fast-inactivation and glycosylation. The 13.9 kb gene that spanned the full-length revealed that 85% of the introns were found to partition the coding region in the homologous position of mammalian Na^+ channels. One of these introns is spliced by a rare U12-type spliceosome conserved in *P. penicillatus* and most

Abstract

Ca^{2+} and Na^{+} channels. Intron positions found in mammalian genes but not the jellyfish gene correspond to highly variable regions which were rich in structural motifs for glycosylation, phosphorylation and binding to cytoplasmic proteins.

Similarity of features between jellyfish and mammalian Na^{+} channels extends from the exon-intron structure of the gene, the different splicing mechanisms, the structure of the cDNA to the electrophysiological properties of recorded currents. Overall, this Ph.D. research strongly suggests that the organization and fundamental composition of the genetic template for Na^{+} channels has undergone few changes since it was laid down in the common ancestors of diploblasts and triploblasts, ~600 million to 1 billion years ago.

This thesis is dedicated to my parents for creating an environment that any prairie kid would like to grow up under; to my dad, the coach who hauled my brothers and I for all those years to early morning practices, rinks and ball fields all over the prairies, and professor –
I'll always be that proud kid heading over to the eighth floor of the Arts Building, University of Saskatchewan to visit my dad.

PREFACE

Dr. Malcolm Sayer is being interviewed for a position at a chronic hospital in the Bronx, New York and asked to describe his previous research experience.[1]

Dr. M. Sayer: *"Earthworms"*

Interviewer board: *"I'm sorry?"*

Dr. M. Sayer: *"It was an immense project. I was to extract one decagram of myelin from four tons of earthworms."*

Interviewer board: *"Really?"*

Dr. M. Sayer: *"Yes. I was on that project for five years. I was the only one to believe in it. Everyone else told me it couldn't be done."*

Interviewer board: *"It can't".*

Dr. M. Sayer: *"I know that now. I proved it."*

I remember a few years ago, there was a table set up outside the main office of the Biology Department, stacked with old Masters and Ph.D. Theses. A small sign in front gave notice of the free offerings to anyone who would haul them away. Weeks later, the stacks were still piled high on the table. I guess free wasn't cheap enough.

Years from now, when they clear out the main office, my thesis will be in amongst the discards with titles such as *"An Exhaustive Study of Myelination in Earthworms"*. I would be curious to see how antiquated my research would appear to someone flipping through this thesis, say, twenty years from now. When I started the Ph.D., PCR-based technology was still in its infancy. I remember when Warren was showing me how to use his first almost brand new, water-cooled PCR machine. I

---

[1] Dialogue from Steven Zillian's screenplay for the movie "Awakenings", Columbia Pictures Industries Incorporated, 1990.

Preface

remember my first sequencing experience -- two days of labor and 100 base pairs of bad sequence. You had to squint to count all the fuzzy bands. Was that three or four "T" in a row? Is that a piece of gook on the X-ray or valid data? Now, sequencing is as easy as taking a role of film for development at the drugstore. On an average day, you can obtain over 850 bp of readable sequence from one sample. It comes back to you all neatly color-coded and annotated in a nifty computer file. Email was a new thing back then. "Web" referred to spiders, not computer networks. We all shared the Mac SE laboratory computer in Warren's lab with its faded mini-computer screen.

I remember discussing with Andy about a possible cloning project of jellyfish sodium channels in 1992. At that point we were not certain that jellyfish would have homologous genes. Why would they? However, soon after, Tim Jegla cloned and expressed jellyfish potassium channels and Peter Anderson had cloned a scyphozoan sodium channel gene. Now it seems more rare for a structural class of mammalian genes to be missing from simple organisms.

This Ph.D. degree has been a humbling, but valuable experience. The process reminds me a lot to the adjustments that I was forced to make as a kid. In a way, it is like pee-wee hockey all over again. "Work hard", "keep your eye off the scoreboard and on the puck", "finish your checks", "keep your head up in the corners", "pass to the open player". Eventually things will bounce your way.

David Spafford, April 1998.

ACKNOWLEDGEMENTS

It has been said in reference to raising children properly that "it takes a village"[1] and it applies equally well to the development of a scientist. I have been fortunate to be surrounded by many very good, hard working scientists in my graduate program. The first person I should mention is Nikita Grigoriev who was my collaborator in the electrophysiological experiments and scientific ideas presented in Chapters 2 and 3. He also was the creator behind a joint project that will lead to a few publications dealing with the structure and function of *Shaker* potassium channels. Not only has Nikita been a mentor in science for me, but over the six years he has been a very close, trustworthy friend. He has been unwavering in his encouragement and support for me.

The other half of my thesis (chapters 4 and 5), could not have been completed without the guidance and support from my co-supervisor Warren Gallin, who patiently has nurtured me since I started as a newbie in molecular biology in 1993. I am also grateful to my head supervisor, Andy Spencer who took me under his wing as a graduate student in 1992.

I spent part of two summers and a nine month stint in Bamfield, and for this I am grateful to the staff at the Bamfield Marine Station who provided research facilities and assisted in collecting and holding jellyfish; I especially thank: Drs. B.M. Olivera and J.M. McIntosh at the University of Utah for supplying *Conus* venoms and purified toxins

[1] Hillary Clinton, First Lady of the United States

Acknowledgments

(Chapter 2); P. Ruben for his helpful discussions concerning the kinetics of inactivation (Chapter 3); P.A.V. Anderson and R.J. Dunn for sharing their experiences concerning the cloning and expression of invertebrate sodium channels (Chapter 4); Curtis Strobeck for allowing me to use their molecular biology hardware from time to time (Chapters 4 and 5); Jeff Goldberg for allowing us to tie up his research groups' 266 MHz Power PC for days at a time for bootstrap analyses (Chapter 5) and Arlin Stoltzfus for his helpful insights regarding alignments of introns/exons between homologous genes (Chapter 5).

I would also like to thank my committee members for their participation, development and evaluation of my research program, Dr. Jeffery I. Goldberg, Dr. Curtis Strobeck, and Dr. Susan M.J. Dunn (Department of Pharmacology). I also thank in advance Dr. Alan Bateson (Department of Pharmacology), Dr. P.A.V. Anderson (University of Florida) and Dr. Frank E. Nargang for sitting on my defense committee as examiners.

I would like to thank the financial assistance provided by: the Province of Alberta in the first year of my degree; the Alberta Heritage Foundation for Medical Research who supported me in the last five years of my degree; the Department of Biological Sciences for the experience and monies provided in teaching assistantships in three terms of my degree; travel grants in 1996 from the Mary Louise Imrie Award and the Department of Graduate Studies and Research, University of Alberta; operating grants from the Natural Sciences and Engineering Research Council of Canada to Dr. A.N. Spencer and Dr. W.J. Gallin.

Acknowledgments

On the personal side, I am lucky to have my sister, Sarah, the antidote to clear my oft-muddled thoughts over the years -- Last and most important, my soulmate and life companion, Monica.

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LIST OF SYMBOLS, NOMENCLATURE AND ABBREVIATIONS

Symbol / Nomenclature / Abbreviation	Description
3' UTR	3' untranslated region; untranslated nucleotide sequence of the mRNA that is downstream from a stop site (TAA, TAG, TGA)
5' UTR	5' untranslated region; untranslated nucleotide sequence of the mRNA that is upstream from the initiation codon (ATG)
5'RACE	rapid amplification of cDNA ends; PCR amplification of the 5' end of a cDNA strand using a template synthesized from the reverse-transcription of mRNA
A	alanine, an amino acid - or - nucleotide base with adenine nucleic acid
B neuron	neuron which is identified in nerve-enriched primary culture of *P. penicillatus* tissues by long, relatively fine processes and bulbous cell body; is electrophysiologically identifiable by characteristic action potential shape and slow recovery from inactivation (half recovery time > 50 ms); contrasts with SMN
B	"not A" nucleotide base (C or G or T/U)
bp	base pair(s), measurement of the length of a DNA strand in numbers of nucleotide bases
C	cysteine, an amino acid - or - nucleotide base with cytosine nucleic acid
Ca^{2+} channel	calcium channel; voltage-gated ion channel, selectively-permeable to calcium ions; belongs to a family of related voltage-gated calcium channel proteins
cDNA	complementary DNA, synthesized by the reverse-transcription of RNA
C_m	membrane capacitance; measured in coulombs of electric charge^{-12} (pF)
D	"not C" nucleotide base (A or G or T/U) - or - aspartate, an amino acid

List of symbols, nomenclature and abbreviations

Symbol / Nomenclature / Abbreviation	Description
D1, D2, D3, D4	membrane-spanning, repeat Domains 1 through 4, generated by duplication
DMSO	dimethyl sulphoxide; solubilizing agent for lipophilic compounds
dNTP	deoxynucleotide (adenosine, guanine, cytosine and thymidine in equal proportions) 5' triphosphate; provides deoxynucleotides needed for generation of a DNA strand in PCR
E	glutamate, an amino acid
EGTA	ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
E_r	reversal potential of ionic current
F	Farad(s); measurement of capacitance (coulombs of electric charge); may be prefixed: nF, pF - or - phenylalanine, an amino acid
G	glycine, an amino acid - or - nucleotide base with guanine nucleic acid - or - prefix: giga (10^6) examples: GHz, GΩ
G_{max}	maximal ion conductance
H	"not G" nucleotide base (A or C or T/U) - or - histidine, an amino acid
HEPES	*N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid
I	ionic current - or - isoleucine
ID1-2, ID2-3, ID3-4	cytoplasmic linkers between domains 1 and 2, 2 and 3 and 3 and 4
I_{max}	maximal ionic current

List of symbols, nomenclature and abbreviations

Symbol / Nomenclature / Abbreviation	Description
I_{test}	peak ionic current amplitude expected on the test pulse response
I-V	ionic current – voltage relationship
K	keto nucleotide base (G or T/U) - or - lysine, an amino acid
K^+ channel	voltage-gated ion channel, selectively permeable for potassium ions; belongs to a family of related voltage-gated potassium channel proteins
K_a	slope factor of the activation curve in millivolts per *e*-fold change
kDa	kiloDalton(s); a unit of molecular mass; numerically a Dalton is equivalent to one-twelfth the mass of a carbon atom
K_i	slope factor of the inactivation curve in millivolts per *e*-fold change
L	leucine, an amino acid
M	amino nucleotide base (A or C) - or - methionine, an amino acid
mRNA	messenger RNA
N	any nucleotide base (A or C or G or T/U) - or - asparagine, an amino acid
neurons[1]	jellfyfish cells that appear to be analogous to mammalian neurons forming electrical and/or synaptic-like connections in a network, some of which for eg. B neurons have a morphology like mammalian neurons
NMG	*N*-methyl-D-glucamine

[1] A definition that applies to the hydrozoan jellyfish, *Polyorchis penicillatus*

List of symbols, nomenclature and abbreviations

Symbol / Nomenclature / Abbreviation	Description
oligo-dT	a short oligonucleotide primer consisting of deoxythymidine nucleotides; used to synthesize a cDNA strand from the 3' poly A tail of mRNA
orthologous genes	genes that are derived from a speciation event
P	proline, an amino acid
P. penicillatus[2]	*Polyorchis penicillatus*, an anthomedusan; a hydrozoan jellyfish; a cnidarian' a coelenterate; a diploblast; a metazoan; an animal; a eukaryote; phylogeny: Eukaryotae – Metazoa – Cnidaria – Hydrozoa – Hydroida – Anthomedusae – Polyorchidae – Polyorchis
paralogous genes	genes that are derived from a duplication event; for eg. voltage-gated Ca^{2+} and voltage-gated Na^{+} channels
PCR	polymerase chain reaction; amplification of DNA between two short oligonucleotide primers using a thermostable DNA polymerase
polyA signal	AAUAAA motif in mRNA transcript 10 to 30 nucleotides upstream from poly A tail; signal for the cleavage of the primary mRNA transcript
polyA site	polyadenylated tail of RNA; a string of adenine nucleotide bases at the 3' end of the untranslated region of the primary mRNA transcript
PpSCN1	a voltage-gated sodium channel gene isolated from *P. penicllatus* (GenBank Accession numbers AF047379 and AF047380)
Q	glutamine, an amino acid
R	arginine, an amino acid - or - purine nucleotide base (A or G)
R_s	series resistance
RT-PCR	polymerase chain reaction of cDNA synthesized from the reverse transcription of mRNA
S	serine, an amino acid - or - strong nucleotide base (C or G)

[2] only the medusa stage of this animal was examined in this dissertation

List of symbols, nomenclature and abbreviations

Symbol / Nomenclature / Abbreviation	Description
sodium channel	voltage-gated ion channel, selectively-permeable to sodium ions; belongs to a family of related voltage-gated sodium channel proteins; physical entity assumed to be responsible for a recorded sodium-selective current; the primary, pore-forming, α subunit
sodium current	voltage-sensitive current selective for sodium ions; voltage-gated sodium channel gene(s) is/are responsible for the current recorded
S1, S2, S3, S4, S5, S6	alpha-helical, hydrophobic, transmembrane segments one though six
S5-P	the extracellular loop between segment five and the membrane-penetrating, pore-forming P segment (short-segments I and II) between segment five and segment six
SMN	neuron which is identified in nerve-enriched primary culture of *P. penicillatus* tissues by large size, some 30-50 μm long with blunt processes up to 200 μm, clear cytoplasm and a nucleus surrounded by membranous structures; reminiscent in shape to epithelial cells; is electrophysiologically identifiable by characteristic action potential shape and slow recovery from inactivation (half recovery time > 50 ms); contrasts with B neuron
STX	saxitoxin; analogue of tetrodotoxin; toxin from marine plankton that selectively blocks sodium channels
T	deoxyribonucleotide base with thymine nucleic acid - or - threonine, an amino acid
TEA	tetraethylammonium
TTX	tetrodotoxin
TTX- insensitive	refers to voltage-gated sodium currents / channels that are not blocked by 100 μM tetrodotoxin; most cnidarian voltage-gated sodium currents belong to this group
TTX-resistant	refers to voltage-gated sodium currents / channels that are blocked by tetrodotoxin concentrations of 1 to 10 μM; mammalian channels SCN5A (heart) and SCN10A (dorsal root ganglion) belong to this group

List of symbols, nomenclature and abbreviations

Symbol / Nomenclature / Abbreviation	Description
TTX-sensitive	refers to voltage-gated sodium currents / channels that are blocked by tetrodotoxin concentrations in the nanomolar range; most voltage-gated sodium currents / channels belong to this group
V	"not T/U" nucleotide base (A or C or G) - or - valine, an amino acid
V_a	voltage of half-activation
V_c	command voltage
Vg	voltage-gated
V_h	holding potential; the potential the recorded cell is held between the application of voltage steps
V_i	prepulse voltage causing half-inactivation
V_{pp}	prepulse voltage, at which the cell membrane is momentarily conditioned before applying a test pulse
W	tryptophan, an amino acid - or - weak nucleotide base (A or T)
Y	pyrimidine nucleotide base (C or T/U) - or - tyrosine, an amino acid

CHAPTER 1 INTRODUCTION

Voltage-dependent sodium channels are responsible for the rapid influx of Na^+ ions that leads to the rapid propagation of action potentials in many excitable cells. Other channel types pass Na^+ ions, including epithelial Na^+ channels (Garty and Palmer, 1997), cotransporters (Turk and Wright, 1997) and ligand-gated channels (Ortells and Lunt, 1995), but they are structurally different and likely have an independent evolutionary origin. Genes coding for voltage-gated Na^+ channels belong to a class of structurally related gene families, including voltage-gated K^+ and Ca^{2+} channels (Goldin, 1995). Sodium and calcium channels consist of four membrane-spanning domains (fig.1-1, top), with each domain containing six membrane-spanning segments (fig. 1-1, middle). Four subunits of potassium channel polypeptide or a single sodium and calcium channel protein fold into a tertiary channel structure with domains predicted to contribute a membrane–penetrating, pore-forming loop (P loop) between segments five and six (fig. 1-1, bottom). In the current model, a few critical residues in these loops (P-loops) provide ionic specificity.

Sodium channels are considered to have evolved from a single domain, of equivalent structure to voltage-gated K^+ channels, by two rounds of duplication (Strong *et al.*, 1993). The duplication pattern of Ca^{2+} and Na^+ channels is evident in the kinship of related domains, where domain pairs I-III and II-IV of the four domain channels share a greater degree of sequence identity (see fig. 1-2) (Strong *et al.*, 1993).

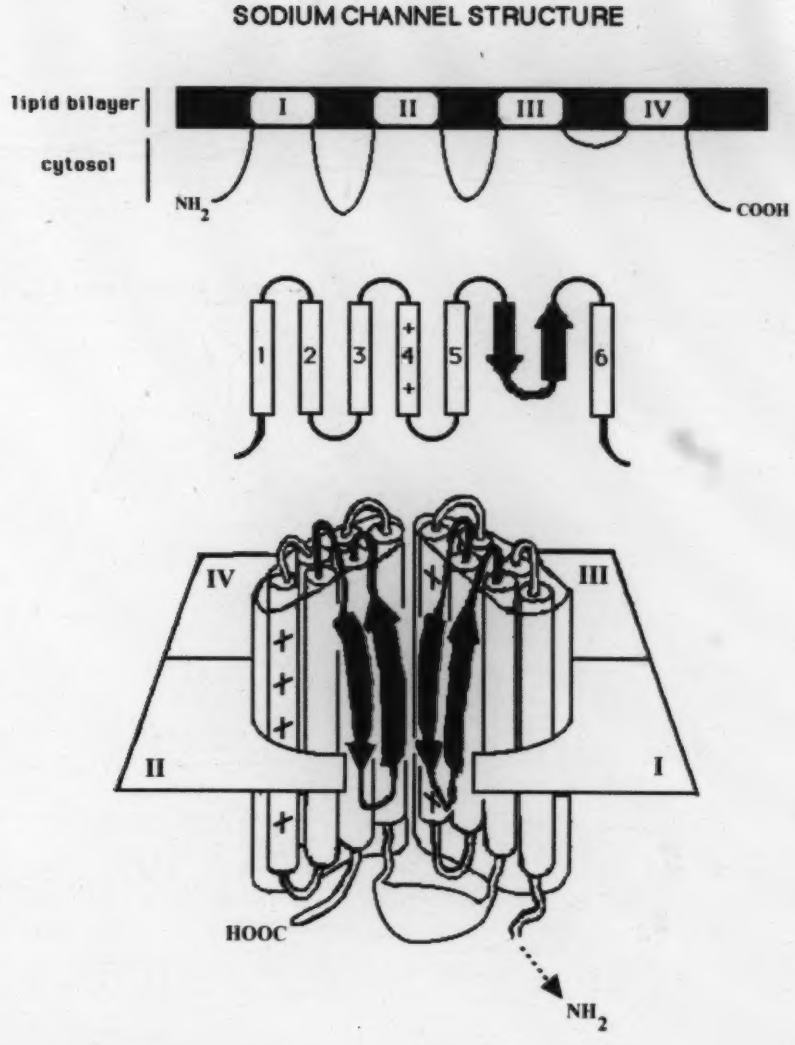

Figure 1-1. Model for the structure of voltage-gated Na^+ channels. General topology of a typical Na^+ and Ca^{2+} channel with four domains (top). Each domain consists of six putative α helical transmembrane segments (middle). All four domains are expected to fold into a tertiary channel structure with each domain contributing a membrane–penetrating, pore-forming loop (P loop) between segments five and six (bottom). Charged residues (+) in the 4th segments are positively-charged and considered to act as voltage-sensors. The structural element for fast inactivation is expected to be a "hinged lid" formed by the cytoplasmic loop between domains III and IV that operates by occluding the pore. Figure modified from Figure 11-27, *in* "Molecular Biology of the Cell Third Edition", (B. Alberts *et al.*, Eds.), p. 534, Garland Publishing, Inc, New York.

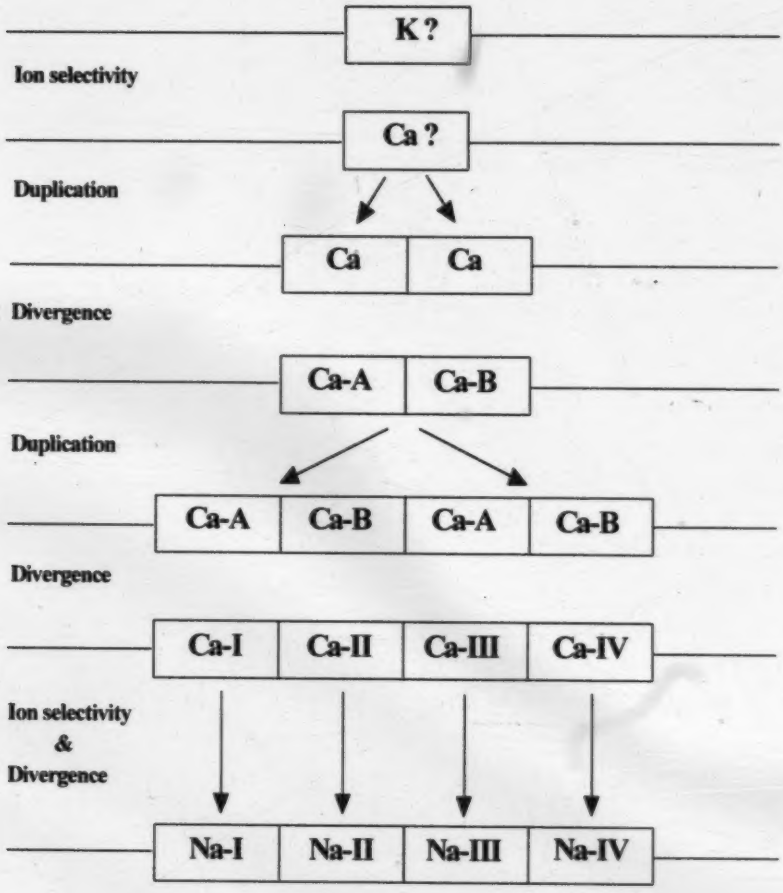

Figure 1-2. Schematic diagram for the predicted evolution of Ca^{2+} and Na^{+} channel domains. The kinship of the channel domains can be traced in Na^{+} and Ca^{2+} channels, given that there was a period of selection following each duplication round, allowing each domain to alter its structure. In the topology of the most parsimonious trees of Na^{+} and Ca^{2+} channels, domains I and III as well as domains II and IV of both channel types appear to be more similar to each other than the domains within each channel type. This suggests that the Na^{+} and Ca^{2+} channels co-evolved from a primordial single-domain channel that had already undergone two rounds of duplication. It is not obvious whether early channels acquired Ca^{2+}-selectivity before or after the duplication rounds events from the K^{+} channels. All Na^{+} and Ca^{2+} channels, including a putative Ca^{2+} channel in yeast, are composed of four homologous domains coming from the same gene. Na^{+} channels likely evolved from a Ca^{2+} channel with four domains since each domain of the Na^{+} channel has a greater structural similarity to the corresponding domain in Ca^{2+} channels than to any other of its own domains or to any other gene. Figure modified from Fig. 8, p. 233 (Strong *et al.*, 1993).

The origins of K^+ and Ca^{2+} channels are thought to be much earlier than Na^+ channels since they have an obligatory and fundamental role to play in all excitable cells in eukaryotes (Hille, 1992). Sodium channels evolved relatively late, apparently from a Ca^{2+} channel ancestor with four domains. Each domain of the Na^+ channel has a greater structural similarity to the corresponding domain in Ca^{2+} channels than to any other of its own domains or to any other protein (Strong *et al.*, 1993). Sodium channels are sufficiently similar to Ca^{2+} channels that a modification of two critical amino-acids in the pore of two domains of the highly conserved selectivity filter (Heinemann *et al.*, 1992) or phosphorylation of protein kinase A sites (Santana *et al.*, 1998) can produce a Ca^{2+} rather than Na^+ selectivity. The appearance of Na^+ channels allowed Na^+ ions to supplant Ca^{2+} ions as the major carrier of the upstroke of the action potential in some cells. Calcium ions can carry the upstroke of the action potential, but since they also have a role in generating intracellular signals, they cannot carry large currents without affecting intracellular signaling pathways (Hille, 1992). Cells are more tolerant to Na^+ influx with an upper limit that may reach greater than 5000 times the concentration of Ca^{2+} ions in cells. Thus the appearance of Na^+-selective channels made it possible for neurons to propagate fast action potentials and mediate high frequency signalling without secondary effects on other cellular processes.

Most evidence suggests that voltage-gated, sodium-selective channels from this superfamily first appeared in an ancestral metazoan. The most balanced synthesis of

current opinions regarding the phylogeny of the early metazoans, namely the Porifera, Placozoa, Mesozoa, Cnidaria, Ctenophora and Platyhelminthes, would place this latter phylum as being basal to all later bilateral metazoans with the former five phyla being sister groups with an unknown "planuloid" ancestor of the Platyhelminthes (Willmer, 1990).

Cnidarians have only two germ layers (diploblastic) and are the simplest extant eumetazoans with a discrete nervous system. Extant cnidarians are structurally similar to fossilized cnidarians in the Ediacarian fauna (Willmer, 1990), suggesting that the features of Na^+ channels and currents are also likely to be reminiscent of an ancestral phenotype. Thus the cnidarian condition should be partially representative of early diversification of voltage-gated channels.

Over the past 20 years, histology, electrophysiology and, more recently, molecular biology, have been used to describe the features of excitable cells, and cellular events that underlie behaviors such as swimming and responsiveness to light, in the hydromedusa, *Polyorchis penicillatus* (fig. 1-3). In this animal, Na^+-dependent action potentials have been described in the excitable, exumbrellar epithelium and swimming motor neurons ("SMNs") and "B" neurons in electrically-coupled networks (fig. 1-4).

Figure 1-3. Mature hydromedusa *Polyorchis penicillatus* (approximately two cm in diameter). Photograph taken in August 1992 from the holding tanks at the Bamfield Marine Station, Bamfield, B.C., Canada.

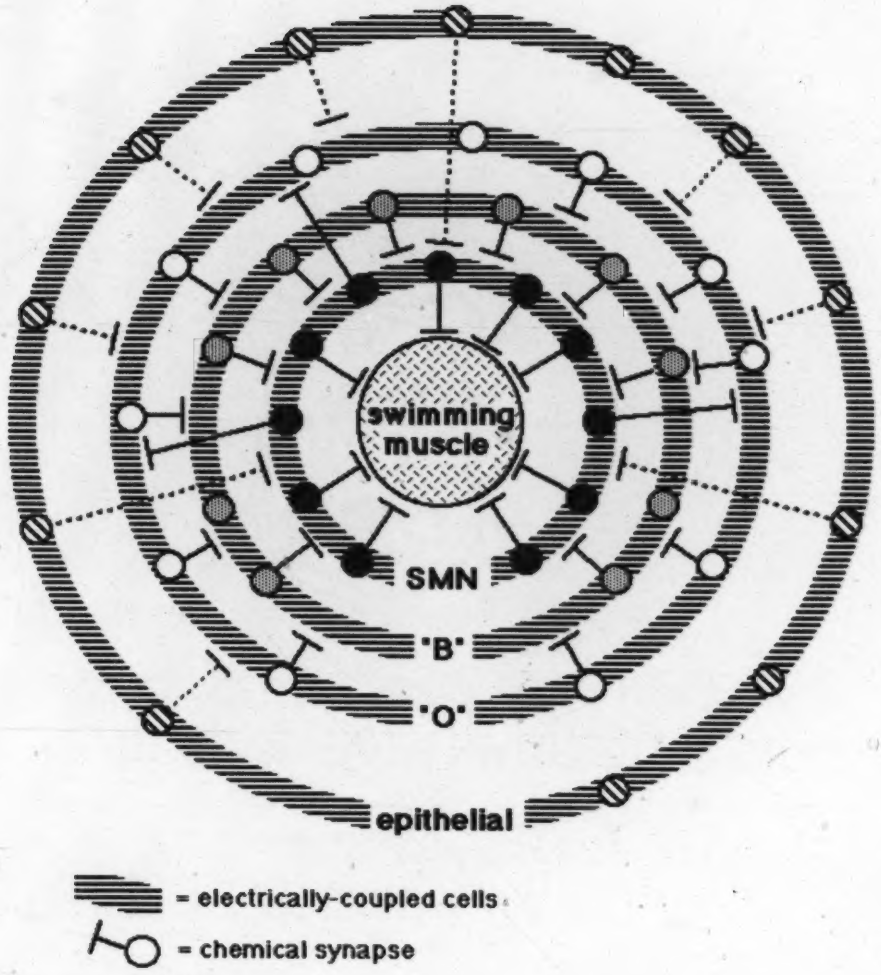

Figure 1-4. Diagrammatic representation of the electrically-coupled networks and potential chemical synapses between identified networks in the hydromedusa *Polyorchis penicillatus*. Putative inhibitory connections are shown with dotted lines. Swimming motor neurons (SMNs) in the inner-nerve ring form a network that innervates the swimming muscle sheet. "B" neurons, located in the outer nerve ring, make excitatory synaptic connections to SMNs and tentacle smooth muscle. "O" neurons, located in ocelli and outer-nerve ring, are photosensitive and make synaptic connections with the "B" and SMN network. Exumbrellar epithelium forms an electrically-excitable sheet that surrounds the outside of the animal. Exumbrellar epithelium, SMNs and B neurons have Na^+-dependent action potentials. Modified from (Spencer and Arkett, 1984).

Pharmacological properties of Na^+ currents in *P. penicillatus*

Sodium currents are also responsible for the rapid rising phase of action potentials in other hydrozoans, such as *Aglantha digitale* (Meech and Mackie, 1993) and *Cladonema* sp. (Anderson and McKay, 1987b), the scyphozoan *Cyanea capillata* (Anderson, 1987a) and ctenophores (Dubas *et al.*, 1988; Hernandez-Nicaise *et al.*, 1980). Even though there is a remarkable consistency in the waveform of voltage-gated Na^+ currents across major phyla, Na^+ currents in cnidarians and ctenophores have unconventional pharmacological properties when compared with Na^+ currents in higher metazoans. In particular, the selective Na^+ current blocker tetrodotoxin (TTX), at 10^{-8} M, blocks greater than 95% of all Na^+ currents in higher metazoans, from flatworms to vertebrates (Hille, 1992), whereas the Na^+-dependent action potentials and Na^+ currents that have been examined to date in excitable cells of lower metazoans and protozoans are minimally affected by TTX, even at concentrations as high as 10^{-4} M (Anderson, 1979). Given the close structural relationship between Ca^{2+} and Na^+ channels (see fig. 1-2), and the basal position of the Cnidaria, it has been suggested that cnidarian Na^+ channels may have intermediate properties between Ca^{2+} and Na^+ channels. In Chapter 2 of this thesis, an extensive description of the pharmacological properties of the Na^+ current in swimming motor neurons (SMNs) of *P. penicillatus* is provided to assist in determining the phylogenetic and functional relationships between early sodium channels. In particular, it is of interest to determine whether a Ca^{2+} channel-like pharmacology is

common to cnidarian Na^+ channels, as suggested by (Anderson, 1987a). The TTX-insensitive Na^+ current that we describe from motor neurons innervating swimming muscle in *Polyorchis penicillatus* has an unusual pharmacological profile for a neuronal Na^+ current but is not altogether like a Ca^{2+} current either. This is shown by the rank order of sensitivity to blockade by di- and trivalent cations ($La^{3+} = Zn^{2+} = Cd^{2+} > Ni^{2+} > Mn^{2+} = Co^{2+} > Ca^{2+} > Ba^{2+} > Mg^{2+}$) and by the low sensitivity of this current to Ca^{2+} channel antagonists like the dihydropyridines, diltiazem and verapamil.

Electrical and kinetic properties of Na^+ channels in *P. penicillatus*

In hydrozoans such as the jellyfish *Polyorchis penicillatus*, every cell in the animal, except perhaps interstitial cells, are capable of generating action potentials. Because the functional roles of these excitable cells are very different, one can expect to find that the excitability properties of the cells are also diverse. The repertoire of action potential shapes, firing frequencies and firing patterns is very rich (Satterlie and Spencer, 1987). Some of this richness in excitability properties is provided by suites of voltage-gated K^+ currents that are known to have diverse electrical and kinetic properties (Rudy, 1988). This physiological and functional diversity of K^+ channels is provided by four subfamilies of genes, *Shaker*, *Shal*, *Shab* and *Shaw* belonging to the *Shaker* superfamily

as well as other K^+ channel gene families (fig. 1-5) (Chandy and Gutman, 1995). It has been shown recently that the *Shaker* gene superfamily is present in *Polyorchis* tissues (Jegla *et al.*, 1995; Jegla and Salkoff, 1997).

Researchers have now recognized some degree of physiological and structural diversity of Na^+ channels (Goldin, 1995). For example, TTX-sensitive and TTX-resistant channels in mammals can be clearly distinguished by a wide range of electrophysiological and pharmacological characteristics (Yoshida, 1994). "Slow" sub-threshold, sodium currents have been identified in Purkinje cells (French and Gage, 1985). Many voltage-gated sodium currents have been characterized by their inactivation kinetics, ranging from very fast (Hille, 1992), to slow (Alonso and Llinas, 1989), to non-inactivating (Taylor, 1993). Sodium channels with different inactivation kinetics can influence action potential shape and play significant physiological roles in mammals (Campbell, 1992).

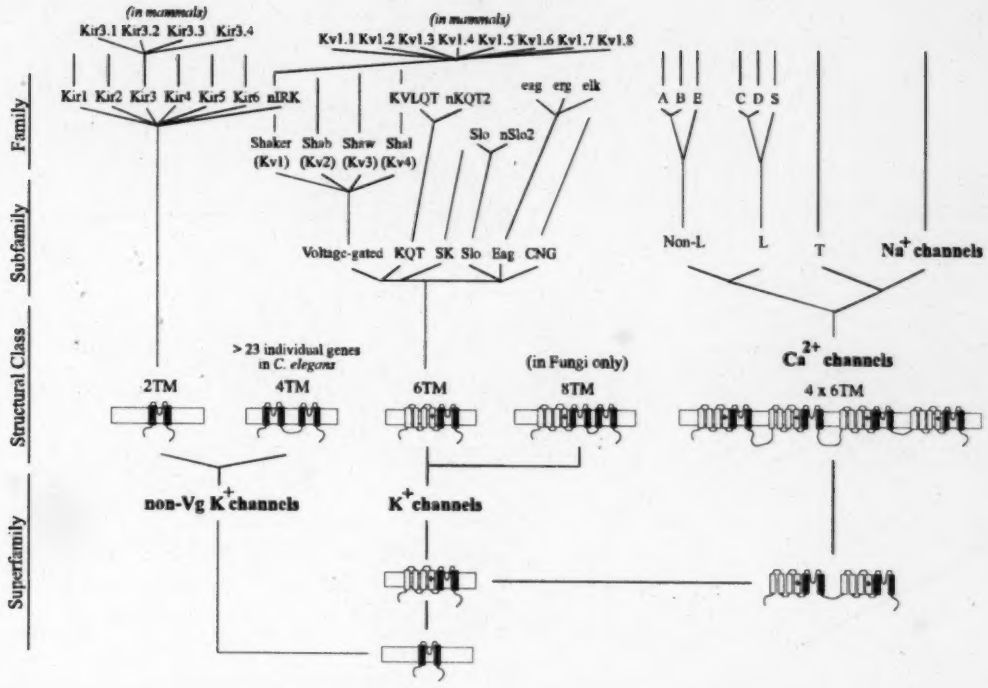

Figure 1-5. A hierarchical classification scheme for the superfamily of ion-selective channels. K^+ channels can be grouped into three structural classes based on their topology: 2TM, 4TM, 6TM and 8TM refer to number of transmembrane segments. Na^+ and Ca^{2+} channels have four repeating domains equivalent to a 6TM K^+ channel (4x6TM). The dark membrane spanning boxes represent the inward rectifying potassium channel that consist of only two membrane spanning segments separated by a pore forming loop. Potassium channels with four, six and eight membrane segments appear to be duplications or modular additions to the basic two transmembrane form. Family members shown in the figure are only mammalian genes. Figure modified from Fig. 1, p. 806 (Wei *et al.*, 1996).

In *P. penicillatus*, at least three different sodium currents are present. There is a slowly activating and slowly inactivating sodium current in the electrically excitable epithelium, which is inhibited by high concentrations of intracellular calcium (Grigoriev and Spencer, 1996). In Chapter 3, the electrical and kinetic properties of Na^+ currents in swimming motor neurons are described. They appear to have rapid, transient Na^+-selective currents that are kinetically similar to Na^+ currents in mammals, despite their unusual insensitivity to classical Na^+ channel agonists and antagonists, including TTX (Spafford *et al.*, 1996). The non-neuronal like morphology of swimming motor neurons and the slow inactivation recory rate of their Na^+ currents indicate that these neurons have properties that are more "cardiac-like" rather than "neuron-like". In contrast, a second neuronal type, the B neuron, has a sodium current with properties that are more similar to most neuronal sodium currents. An argument can be made that the non-neuronal properties of the "cardiac-like" current in swimming motor neurons have probably arisen by convergence since in both cases (SMNs and cardiac muscle) spreading of excitatory waves in a closed network is prone to re-entry. In bot the heart and network of SMNs, a slow recovery from inactivation prevents re-entry and disruption of the rhythmic input that leads to synchronous contractions of the heart and jellyfish bell, respectively (Fye, 1987).

Structure of a putative sodium channel protein in *P. penicillatus*

In order to determine the structural basis that may underlie the unusual pharmacological properties of hydrozoan Na^+ currents (Chapter 2) or the basis for the differences between the electrical and kinetical properties of currents in swimming motor neurons (SMNs) and B neurons (Chapter 3), a full-length sodium channel cDNA, PpSCN1 was cloned from the hydrozoan jellyfish, *Polyorchis penicillatus* and described in Chapter 4. Comparisons of PpSCN1 with aligned sequences of other sodium channels, reveal that many of the residues that are considered to be critical for ionic selectivity, voltage sensing, and binding of TTX and lidocaine in mammals differ in the Cnidaria. PpSCN1 is the only α subunit presently available to account for sodium channel currents in SMNs, B neurons and exumbrellar epithelial cells in *P. penicillatus*. The apparent absence of structural diversity of α subunits suggests that the functional diversity of sodium channels in *P. penicillatus* may depend on accessory proteins or post-translational modification of α subunits, or that there are other sodium channel families present that are so structurally distinct as not to be isolated by the methods used in this study.

Genomic structure of a sodium channel gene in *P. penicillatus*

Many of the Na^+ channels isolated in mammals and fruit fly have multiple introns that are spliced out of the final mRNA transcript. Some of these genes contain alternative or optional exons, providing a rich source of genetic diversity. The complete genomic region spanning the cDNA of PpSCN1 was sequenced and compared with mammalian and fruit fly genes in a detailed comparative analysis described in Chapter 5. No alternative spliced variants were apparent in the jellyfish gene. Most of the introns were found to be conserved between Na^+ channel genes in *P. penicillatus* and mammals, with a significant percentage even shared with Ca^{2+} channels. The conservation extends to the details of intron splice sites, including a recently discovered U12-type splice site that employs a rarely used set of small ribonucleoproteins. Regional differences in Na^+ channels appear to correspond to potential adaptive roles among channel types, for example, phosphorylation by protein kinase A, glycosylation and alternative splicing.

Overview and organization of the thesis

In this thesis, the structure, function and diversity of jellyfish Na^+ channels is explored using molecular biological and electrophysiological techniques. The thesis proceeds in reverse order to the fate of the gene in the cell, which would be, the organization of a gene on the chromosome (Chapter 5), transcription and predicted protein structure (Chapter 4) and apparent characteristics of the protein from current recordings of identified cells *in vitro* (Chapter 2 and 3).

A comparative approach, such as this, provides valuable insights into the understanding of the structure and function of voltage-gated Na^+ channels. Functional comparisons between jellyfish and mammalian currents, and alignments of their genes underscore possible structural-functional similarities and differences. Given the long evolutionary history that separates cnidarians and mammals, it provides an opportunity to evaluate the tolerance of structural features to evolutionary change, and hence to adaptive changes taken by different channel types. Remarkable conservation of the mammalian Na^+ channel gene and cDNA transcript in jellyfish, and the conservation of electrical and kinetical properties and pore selectivity to monovalent cations, shows that the organization and fundamental composition of the genetic template for Na^+ channels has undergone few changes since it was laid down in the common ancestors of diploblasts and triploblasts, ~600 million to 1 billion years ago (Morris, 1993).

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CHAPTER 2 PHARMACOLOGICAL PROPERTIES OF VOLTAGE-GATED SODIUM CURRENTS IN MOTOR NEURONS FROM A HYDROZOAN JELLYFISH POLYORCHIS PENICILLATUS[1,2]

INTRODUCTION

The TTX-insensitive sodium current recorded from motor neurons in the scyphozoan jellyfish *Cyanea capillata* shows a pharmacological profile that is more similar to that expected of calcium channels (Anderson, 1987). This finding appears to support the view of (Hagiwara, 1983) that calcium channels are phylogenetically more ancient, since they are diverse and are present in the most 'primitive' organisms. Hille (1984) further developed this theory by proposing that high-velocity conduction, and the associated high current density required, could not be supported by calcium action potentials because of the resulting calcium toxicity. Selection for sodium-permeable channels would therefore be very strong in cells specialized for long-distance, high-frequency signalling. Domain homology between sodium and calcium channels further

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[1] A version of this chapter has been published. Spafford, J.D., Grigoriev, N.G. and Spencer, A.N. (1996) *Journal of Experimental Biology* 199:941-948.

[2] This research was a collaborative effort between Spafford and Grigoriev.

demonstrates that calcium channels were the likely immediate ancestral molecules for sodium channels (Strong *et al.*, 1993). Here, we give an extensive description of the pharmacological properties of a sodium current from an organism that is deeply rooted in the phylogenetic tree. Thus, a study like this assists in determining the phylogenetic and functional relationships between channels carrying the inward currents responsible for propagation of action potentials. In particular, we are interested in determining whether a calcium-channel-like pharmacology is common to such sodium channels that are reminiscent of the ancestral prototype. The TTX-insensitive sodium current that we describe from motor neurons innervating swimming muscle in *Polyorchis penicillatus* has an unusual pharmacological profile for a neuronal sodium current but does not exhibit any specific properties expected for calcium currents.

MATERIALS AND METHODS

Cell culture

Medium-sized jellyfish *Polyorchis penicillatus* (Eschscholtz) (diameter 20-30 mm) were collected in Bamfield and Grappler Inlets (Vancouver Island, British Columbia, Canada) and maintained at approximately 10°C in flow-through seawater aquaria. Cells were cultured using a method from (Przysiezniak and Spencer, 1989) with

slight modifications. Cells were exposed to 1000 units of collagenase per ml of artificial sea water for a shorter period (1 h instead of 4-5 h), with agitation. Recordings were made using cell cultures that were no more than 3 days old.

Swimming motor neurons were identified by their large size (soma 30-50 μm long, with processes up to 200 μm long), clear cytoplasm and a nucleus surrounded by membranous structures. They have a characteristic action potential that is identifiable *in vitro* and *in vivo*. Major features of the sodium currents, such as time-to-peak, peak current, voltage-dependence, time course of responses and pharmacological responsiveness to drugs, did not change with the age of culture.

Whole-cell recordings

Whole-cell, tight-seal recordings were made using electrodes of borosilicate glass tubing (TW-150-4, World Precision Instruments), with resistances of 1-2 MΩ when filled with electrode solutions. Recordings were made at room temperature (approximately 20° C) with an Axopatch-1D amplifier (Axon Instruments), lowpass-filtered at 3 kHz using a four-pole Bessel filter, and digitized using a Labmaster TL-125 acquisition board (Axon Instruments).

Cultures were viewed under phase contrast with a Nikon Diaphot inverted microscope. Fine alignments of the microelectrode near the cell surface were made using a piezoelectric driver (Burleigh). Stimulus control, data acquisition and analysis were performed using pCLAMP 6.0 software (Axon Instruments) on a 486-based (PC) personal computer.

Leakage and capacitative currents were subtracted using –P/4 or –P/5 protocols (pCLAMP 6.0), from a holding potential of –80 mV, before test pulses eliciting active responses were applied. Series resistances (R_s) were optimally compensated to minimize voltage errors (R_s compensation was usually set to values of 80% or more). Membrane capacitance (C_m) and R_s values were obtained by minimizing the capacitative transient in response to a hyperpolarizing voltage step. Only recordings that met the following criteria were used in the analysis. (1) Peak uncompensated current was >2 nA; R_s was 2-4 MΩ before compensating for R_s. (2) C_m and R_s did not change by more than 10% before and after drug application, and recovery after drug application was >70 %. (3) Leakage resistances were in the range 100 MΩ to 0.5 GΩ. (4) After series compensation, sodium currents reached their peak within 800 μs using test pulses with voltage steps from –80 mV to +10 mV.

Pharmacological agents were microperfused in bath solution dispensed from a manifold with a dead volume of less than 1 μl. Gravity flow was controlled by electromechanical pinch valves and the rate of flow provided complete solution exchange in 2 s. Test pulses were applied every 30 s after drug application. As soon as a current

tracing superimposed on the previous trace this was recorded as the experimental trace (saturation), and washing was started. The maximal drug effect was obtained from 30 s to 2 min after drug application. In all figures where the "before" and "after" drug application traces do not superimpose, the trace having a smaller peak current is the trace obtained after washing.

Use-dependent blockade was tested for lidocaine, procainamide and verapamil. In use-dependent blockade, the rate of block tends to be proportional to the number of channels open (Hille, 1992). To test for the use-dependence of these drugs, a hyperpolarizing prepulse (-120 mV for 20 ms) or a depolarizing prepulse (-40 mV for 40 ms) was applied immediately before the test pulse.

Solutions

All culture and recording solution were filtered through cellulose acetate membrane cartridges with a pore size of 0.2 μm before use. Artificial sea water , containing (in mM): NaCl, 376; Na_2SO_4, 26; $MgCl_2$, 41.4; $CaCl_2$, 10; KCl, 8.5; HEPES hemisodium salt, 10; and gentamycin sulphate (50 mg per litre). The pH of this solution was 7.5. Na^+-free electrode solutions, containing (in mM): $MgCl_2$, 2; $CaCl_2$, 1; Hepes-free acid, 10; EGTA, 11; $CsCl_2$, 400; CsOH, 30; and TEA-Cl, 20, were titrated to pH 7.5 with KOH. Standard bath solutions were calcium-free (in mM): NaCl, 395; $MgCl_2$, 50;

N-methylglucamine-HCl, 30; Hepes sodium salt, 10, and were titrated to pH 7.5 with HCl. All outward currents were totally blocked within 5 min after breakthrough, and inward calcium currents were eliminated since the external solution was Ca^{2+}-free. The total divalent ion concentration was kept constant in solutions containing Ba^{2+} or Ca^{2+} by adjusting the $MgCl_2$ concentration.

Drugs were purchased from Research Biochemicals International and were prepared daily in standard bath solution. Stocks of lipophilic compounds were prepared daily in fresh dimethylsulphoxide (DMSO) solution. Final working DMSO concentrations were 0.04-0.5 % (v/v). Control responses with DMSO at the above bath concentrations were subtracted from drug responses. Tetrodotoxin was prepared from a stock solution in acetate buffer at pH 4.5 (1 mg ml^{-1}). Numerical and graphical data are presented as means ± S.E.M.

RESULTS

The quantitative data for all the chemical agents examined are given in Table 2-1.

Table 2-1. Quantitative summary of the effects of drugs on sodium currents in *Polyorchis penicillatus* motor neurons

Drug	Dose (μM)	Effect@ (% ± S.E.M.)	N
Sodium channel antagonists:			
Tetrodotoxin	100	3.26 ± 4.10	6
Lidocaine	1000	40.95 ± 1.19	9
	3000	67.67 ± 1.20	7
Procainamide	1000	23.58 ± 1.25	4
Veratridine *	100	30.18 ± 9.47	4
Calcium channel antagonists:			
Dihydropyridines			
Nicardipine *	40	13.86 ± 2.22	9
	70	26.38 ± 1.15	4
	100	55.24 ± 7.03	7
Nitrendipine *	100	16.66 ± 0.60	5
	200	37.35 ± 0.51	3
Nimodipine *	100	8.07 ± 4.66	3
Nifedipine *	100	-0.40 ± 1.79	5
	500	4.38 ± 0.48	3
(+)BayK 8644 *	100	25.29 ± 2.00	9
(-)BayK 8644 *	100	-4.63 ± 0.61	9
Benzothiazepines			
Diltiazem	50	13.73 ± 4.54	3
	100	29.26 ± 2.64	5
	500	53.23 ± 0.54	9
	1500	64.49 ± 3.82	6
Phenylalkylamines			
Verapamil	100	14.97 ± 1.31	6
	500	24.17 ± 3.74	4
	1500	38.12 ± 0.12	4
Calmodulin antagonists:			
W7	100	17.49 ± 1.75	4
Calmidazolium	20	15.37 ± 1.74	9
Non-specific antagonist:			
Capsaicin	1	14.70 ± 4.43	3
	5	73.53 ± 8.76	3
	25	89.91 ± 7.62	3

*Control responses in the presence of DMSO were subtracted from values recorded in the presence of drugs.

@ Percentage inhibition of peak current due to drug action. Peak current was generated using voltage steps of 10 ms duration to +10 mV from a holding potential of –80 mV.

Effect of classical sodium channel blockers and modifiers (TTX, lidocaine, procainamide, veratridine)

The sodium current from 'swimming motor neurons' was insensitive to TTX at concentrations from 1 to 100 μM (Fig. 2-1; Table 2-1). The local anaesthetics lidocaine and procainamide caused partial, tonic blockade of sodium currents at concentrations in the millimolar range and did not exhibit use-dependent blockade (Fig. 2-1; Table 2-1). Veratridine, at 100 μM, produced partial blockade of the sodium current without affecting inactivation properties (Fig. 2-1; Table 2-1).

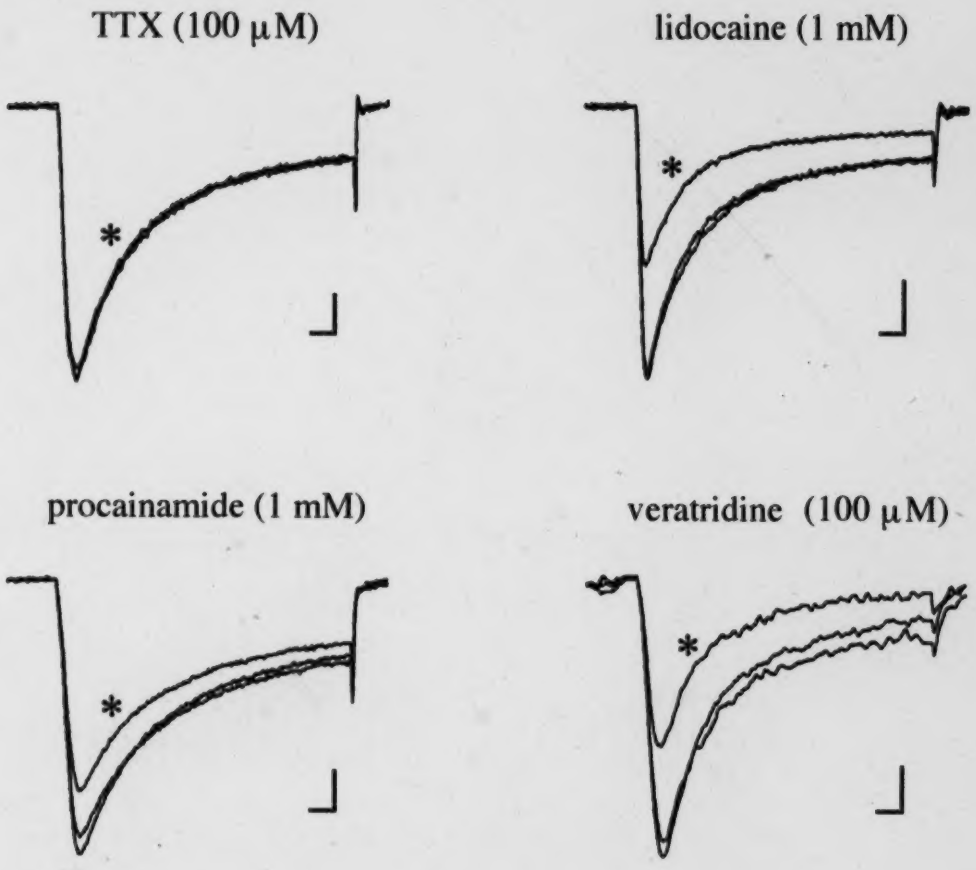

Figure 2-1. Effects of sodium channel antagonists (TTX, lidocaine, procainamide) and a modifier (veratridine) on sodium current in swimming motor neurons of *P. penicillatus*. Tracings marked with an asterisk represent drug effects. Tracings are also shown before drug application and after complete washing. In this and all other figures, where the 'before' and 'after' drug application traces do not superimpose, the trace having a smaller peak current is the trace obtained after washing. Responses were generated using voltage steps of 10 ms duration to +10 mV from a holding potential of –80 mV. Vertical and horizontal scale bars represent 1 nA and 1 ms, respectively.

Effect of di- and trivalent cations

Fig. 2-2 shows the effects of La^{3+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Mn^{2+} and Co^{2+} on Na^+ current at bath concentrations of 3 mM and 1 mM. La^{3+}, Zn^{2+} and Cd^{2+} were the most potent blockers, followed by Ni^{2+} then Mn^{2+} and Co^{2+}. All these cations produced depolarizing shifts in the conductance-voltage curves, with La^{3+}, Zn^{2+} and Cd^{2+} causing the greatest shifts of +10, +12 and +10 mV respectively (data not shown).

Ca^{2+} and Ba^{2+} were less effective cationic blockers. At concentrations of 10, 30 and 50 mM, Ca^{2+} was a more potent blocker than Ba^{2+} (Fig. 2-3). Ca^{2+} produced a noticeable depolarizing shift in the current-voltage curve whereas Ba^{2+} had little, if any, effect. Charge screening effects (Hille *et al.*, 1975) should not apply here, since the concentration of divalent cations was kept constant by substitution with Mg^{2+}. The order of blocking effectiveness for all these cations is: $La^{3+} = Zn^{2+} = Cd^{2+} > Ni^{2+} > Mn^{2+} = Co^{2+} > Ca^{2+} > Ba^{2+} > Mg^{2+}$.

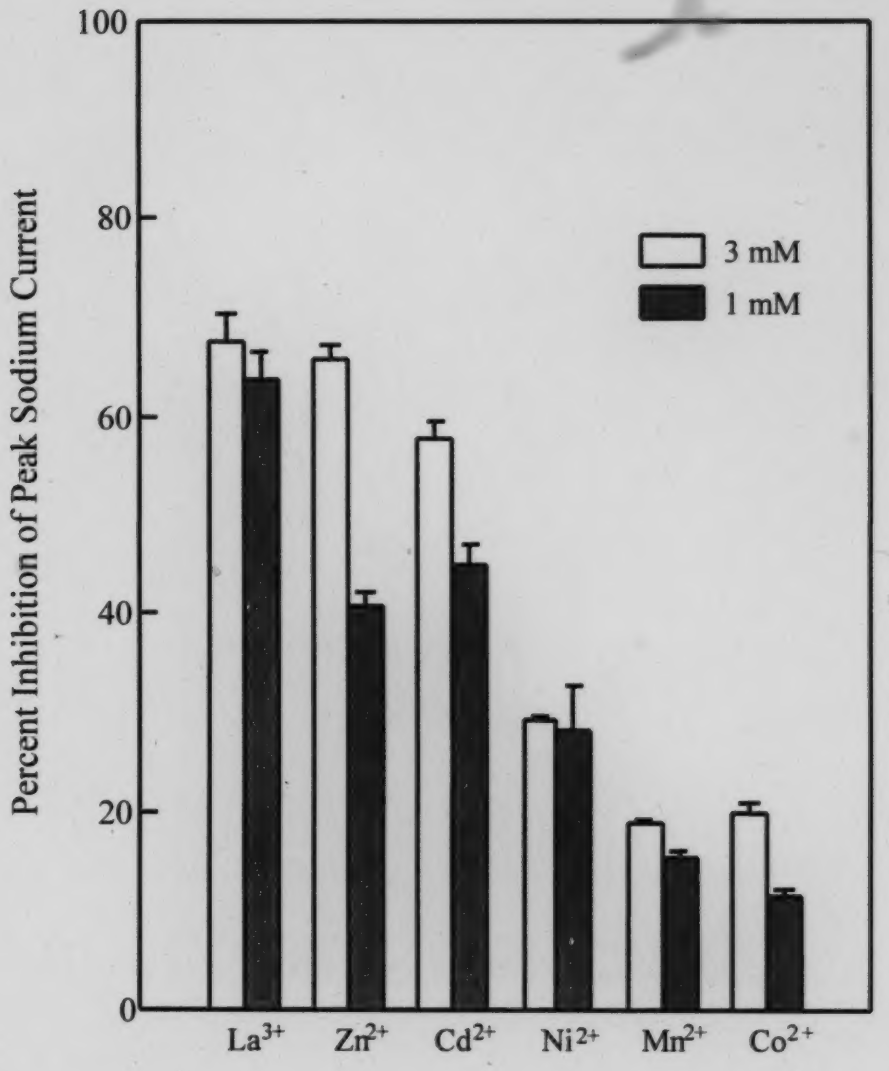

Figure 2-2. The inhibitory effects of di- and trivalent cations on sodium current at 1 mM (shaded bars) and 3 mM (unshaded bars) on sodium current in swimming motor neurons of *P. penicillatus*. Data were obtained from the peak of sodium currents generated using voltage steps of 10 ms duration to +10 mV from a holding potential of –80 mV. Bars represent S.E.M. Numbers of cells used at 1 mM and 3 mM respectively: La^{3+} (9,5), Zn^{2+} (10,12), Cd^{2+} (4,7) Ni^{2+} (5,4) Mn^{2+} (5,5) and Co^{2+} (6,5).

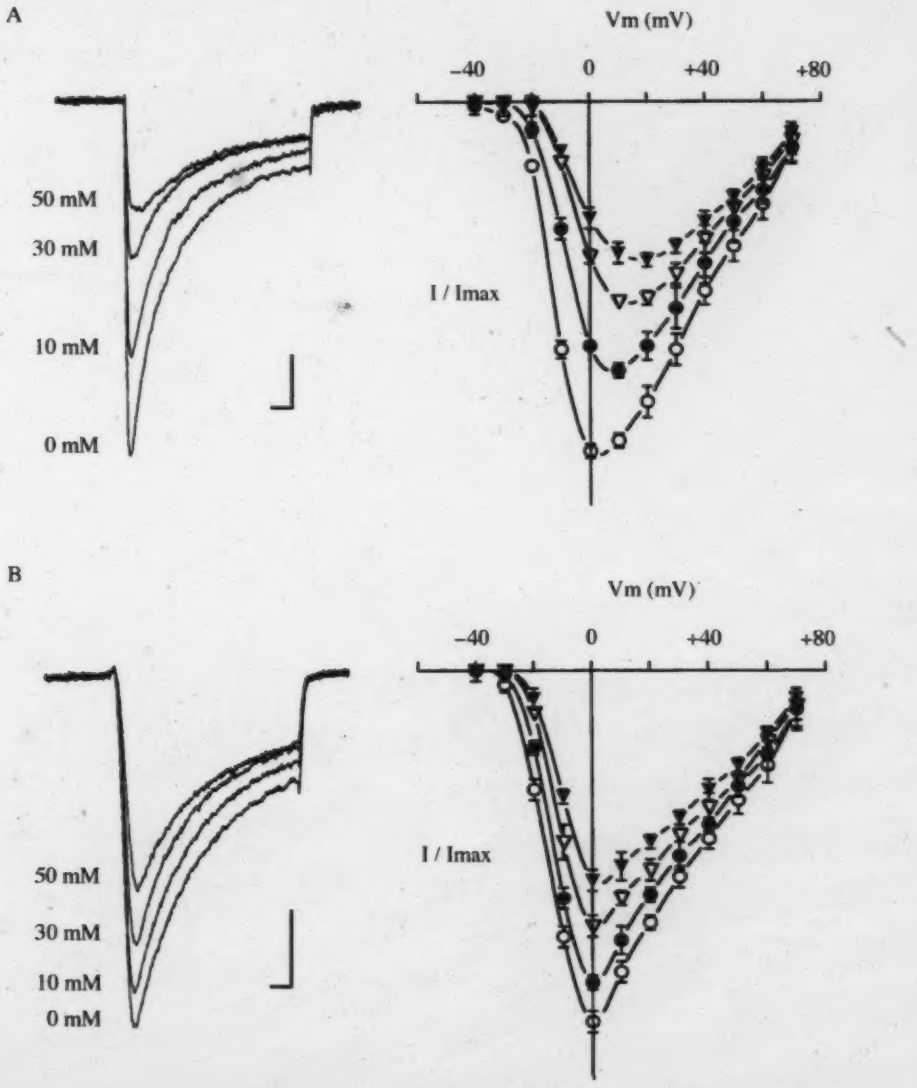

Figure 2-3. Effects of 10, 30 and 50 mM Ca^{2+} and Ba^{2+} on sodium current in swimming motor neurons of *P. penicillatus*. Left-hand side: Sodium current tracings in Ca^{2+} (A) and Ba^{2+} (B) solutions generated using voltage steps of 10 ms duration to +10 mV from a holding potential of –80 mV. Right-hand side: current-voltage relationships of n=3 using Ca^{2+} (A) and Ba^{2+} (B) at concentrations of 0 mM (open circles), 10 mM (filled circles), 30 mM (open triangles) and 50 mM (filled triangles). Currents are normalised to peak control currents. Responses were generated using voltage steps from –40 mV to + 70 mV in 10 mV increments. The holding potential was –80 mV. Vertical and horizontal scale bars represent 1 nA and 1 ms, respectively. Error bars represent S.E.M.

Effect of calcium channel antagonists (dihydropyridines, verapamil, diltiazem) and an agonist (-)Bay K 8644

Of the dihydropyridines, nicardipine had the greatest inhibitory effect (Table 2-1), but even at 70 μM it only produced a 26% decrease in peak current (Fig. 2-4; Table 2-1). Nitrendipine at 100 μM reduced the current by 17%, while nimodipine and nifedipine at 100 μM had no significant effect (when the inhibitory effect of DMSO in the vehicle solution was taken into account). The antagonistic enantiomer (+)Bay K 8644, at 100 μM, inhibited peak sodium current by 25%, while the agonistic (-) enantiomer had not significant effect (Fig. 2-4; Table 2-1). The solubilising agent DMSO at 0.1% (v/v) also inhibited the current by approximately 10% (n=6).

Diltiazem and verapamil blocked the sodium current in a dose-dependent manner. At 500 μM, they produced 53% and 24% decreases, respectively, in current amplitude (Fig. 2-5; Table 2-1). There was no evidence of use-dependent blockade by verapamil.

Effect of calmodulin inhibitors W7 and calmidazolium

Neither W7 (100 μM) nor calmidazolium (20 μM) was a very effective blocker of sodium current (Fig. 2-5; Table 2-1).

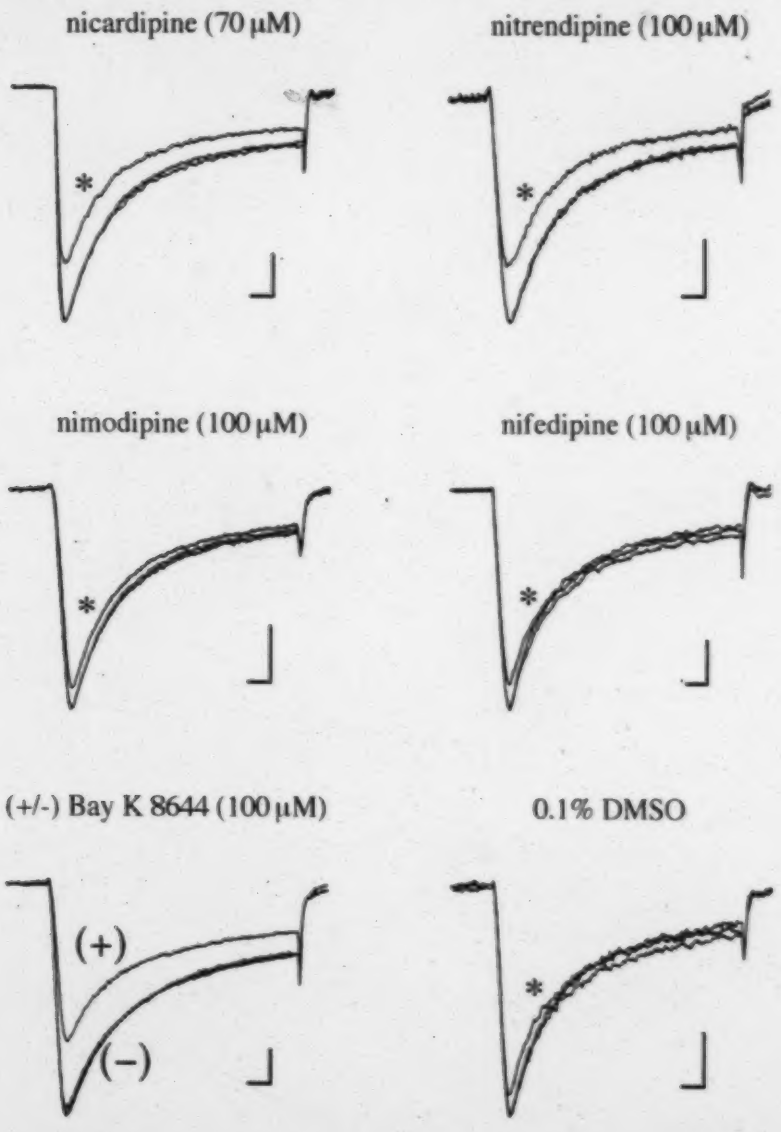

Figure 2-4. Effects of dihydropyridines and the solubilizing agent dimethylsulphoxide (DMSO) on sodium current in swimming motor neurons of *P. penicillatus*. Tracings marked with an asterisk represent drug effects. Tracings are also shown before drug application and after complete washing. For Bay K 8644, the + and – are marked; note the effect of (-)Bay K 8644 was no different from the control or wash traces for both enantiomers. Responses were generated using voltage steps of 10 ms duration to +10 mV from a holding potential of –80 mV. Vertical and horizontal scale bars represent 1 nA and 1 ms, respectively.

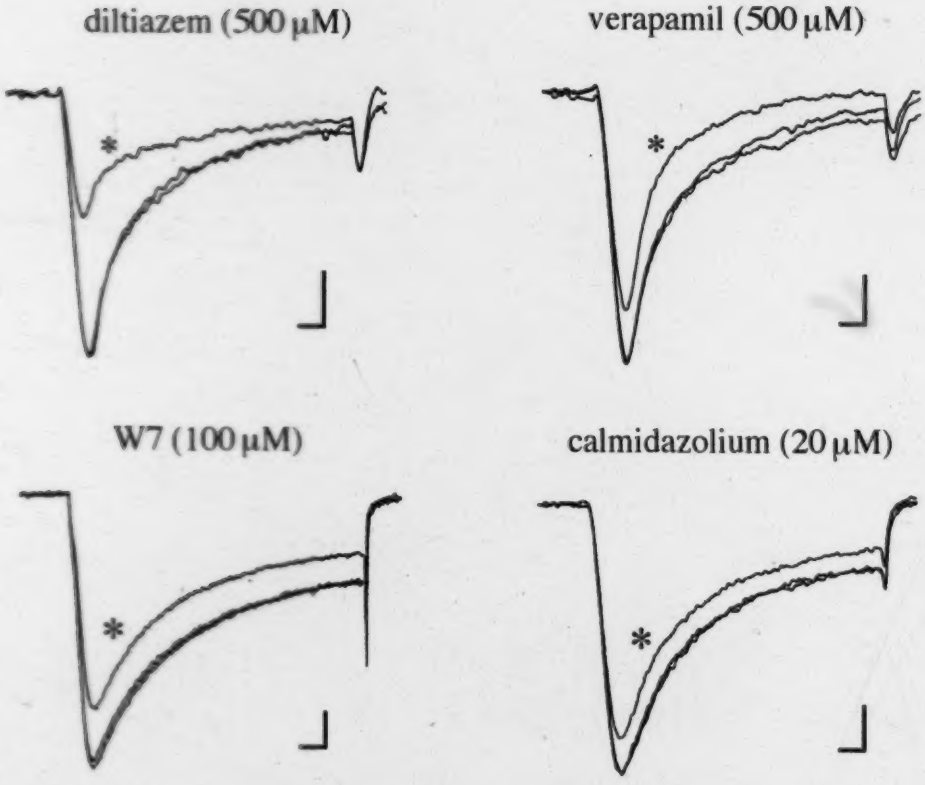

Figure 2-5. Effects of antagonists of L-type calcium channels (diltiazem, verapamil) and calmodulin antagonists (W7 and calmidazolium) on sodium current in swimming motor neurons of *P. penicillatus*. Tracings marked with an asterisk represent drug effects. Tracings are also shown before drug application and after complete washing. Responses were generated using voltage steps of 10 ms duration to +10 mV from a holding potential of –80 mV. Vertical and horizontal scale bars represent 1 nA and 1 ms, respectively.

Effect of Conus neurotoxins

The following *Conus* venoms and peptides were applied to motor neurons: crude venom (1 mg ml^{-1}) from *C. quercinus*, *C. textile* and *C. geographicus*; purified peptides, μ-conotoxin GIIA (1 μM), μO-conotoxin MrVIA (0.5 μM) and the ω-conotoxins GVIA (1 μM) and MVIIC (1 μM). These *Conus* venoms and peptides had no effect (data not shown).

Effect of capsaicin

The only drug examined to date which was active at comparatively low concentrations was capsaicin (*trans*-8-methyl-N-vanillyl-6-nonemide), which produced a rapid and noticeable blockade of sodium current at a concentration of 5 μM (Fig. 2-6A; Table 2-1). At 25 μM the current was reduced by 90 %. However, the effect of capsaicin is likely not specific since outward potassium currents were also strongly inhibited at higher concentrations (300 μM) (Fig. 2-6B).

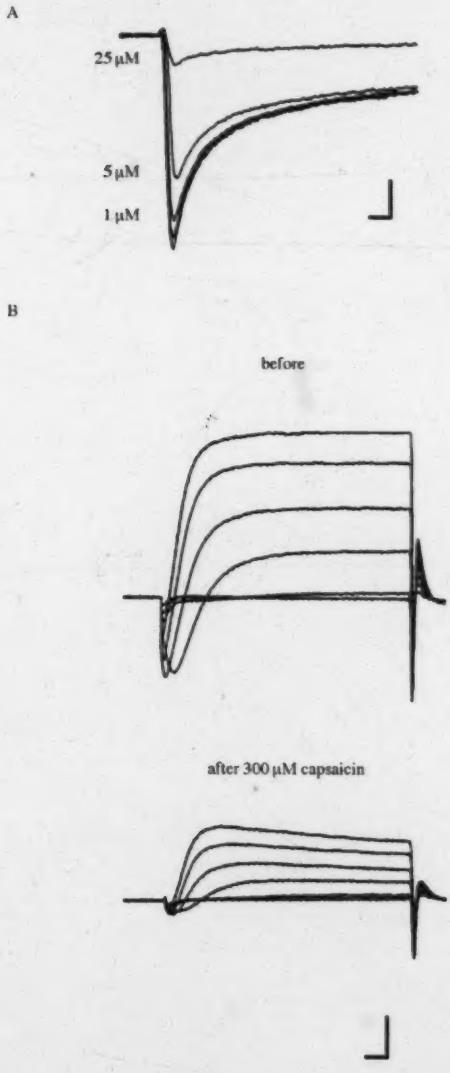

Figure 2-6. Effect of capsaicin on sodium current in swimming motor neurons of *P. penicillatus*. (A) Effects of 1, 5 and 25 μM capsaicin on sodium current. Tracings are also shown before drug application and after complete washing. Responses were generated using voltage steps of 10 ms duration to +10 mV from a holding potential of –80 mV. (B) Effects of capsaicin (300 μM) on total ionic current. Upper traces: before application of capsaicin. Lower traces: after application of capsaicin. Responses were generated using voltage steps from –40 mV to +60 mV in 20 mV increments. The holding potential was –80 mV. Vertical and horizontal scale bars represent 1 nA and 1 ms, respectively.

Structural differences in jellyfish α subunits may be responsible for the lack of effect of typical sodium channel antagonists outside of capsaicin. Alternatively, some of the uncharacteristic responses may be contributed by the unusual composition of lipid membranes (Schetz and Anderson, 1993) or unusual sugar moieties on glycosylated residues (Schetz and Anderson, 1995) expected to be present on cnidarian channels *in vivo*.

DISCUSSION

Categorization of sodium channels according to TTX-sensitivity

On the basis of their susceptibility to tetrodotoxin, three classes of sodium currents have been described: TTX-insensitive (100 μM), TTX-resistant (1-10 μM) and TTX-sensitive (nanomolar range) (Anderson, 1987). Our data clearly show that the sodium current of jellyfish swimming motor neurons can be classified as TTX-insensitive since concentrations as high as 100 μM did not block these currents. These data confirm the finding of Anderson, (1979) that propagation of action potentials in the swimming motor neuron network of *Polyorchis penicillatus* is not affected by TTX at concentrations up to 125 μM. An analogue of TTX, saxitoxin, at 25 μM also failed to block sodium currents (Przysiezniak, 1993).

TTX-insensitive sodium currents have been described in other cnidarians and ctenophoran cells (Anderson, 1987; Anderson and McKay, 1987; Dubas *et al.*, 1988; Meech and Mackie, 1993), while the only truly voltage-gated sodium current recorded from a protozoan (*Actinocoryne contractilis*) is also TTX-insensitive (Febvre-Chevalier *et al.*, 1986). Thus, although TTX insensitivity or resistance is relatively rare throughout the protostome and deuterostome lineages (< 5% of all species, Hille (1987), it appears to be a common feature of lower metazoan sodium channels.

In mammals TTX-resistant channels have been described from cardiac muscle cells, embryonic skeletal muscle and denervated skeletal muscle (Cohen *et al.*, 1981; Gonoi *et al.*, 1985; Pappone, 1980). These TTX-resistant channels have a characteristic order of effectiveness for di- and trivalent cation blockade. For example, in canine Purkinje fibre sodium channels this order is: $Zn^{2+} > Cd^{2+} = La^{3+} > Ni^{2+} \geq Mn^{2+} > Co^{2+} > Ba^{2+} = Mg^{2+} = Ca^{2+}$ (Hanck and Sheets, 1992), which is very similar to the order of potency ($La^{3+} = Zn^{2+} = Cd^{2+} > Ni^{2+} > Mn^{2+} = Co^{2+} > Ca^{2+} > Ba^{2+} > Mg^{2+}$) observed in this study. The TTX-insensitive sodium current recorded from neurons of the *Cyanea capillata* motor nerve-net (Anderson, 1987) showed a similar Cd^{2+} sensitivity to that recorded here for *Polyorchis penicillatus* motor neurons. It should be noted that Cd^{2+}, at much lower concentrations (0.3 mM) than were used in this study, completely blocks calcium currents in the same *Polyorchis penicillatus* neurons as were used in this study (Przysiezniak and Spencer, 1992). Calcium current in *Cyanea capillata* motor neurons was blocked by 2 mM Cd^{2+} (Anderson, 1989).

The high susceptibility of TTX-resistant channels to blockade by Zn^{2+} and Cd^{2+} is not apparent in mammalian brain and skeletal muscle TTX-sensitive channels (Frelin *et al.*, 1986). Structure / function analyses of the pore-forming α subunit in sodium channels have shown that, in short segment 2 of domain I, a critical cysteine residue in cardiac sodium channels and a tyrosine or phenylalanine residue in a similar position in brain and skeletal sodium channels are critical residues of the TTX receptor site (Sather *et al.*, 1994). A tyrosine to cysteine mutation in skeletal muscle sodium channels and a phenylalanine to cysteine mutation in brain sodium channels substantially lowers TTX sensitivity and creates a high-affinity binding site for Zn^{2+} and Cd^{2+} (Backx *et al.*, 1992; Heinemann *et al.*, 1992a). Thus, the region that is responsible for high sensitivity to TTX also produces a lower Zn^{2+} and Cd^{2+} binding affinity.

Ca^{2+} blockade of sodium current

Ca^{2+} blocked *Polyorchis penicillatus* neuronal sodium currents (Fig. 2-3) in a dose-dependent fashion, at concentrations similar to those reported for canine Purkinje fibre sodium currents (Hanck and Sheets, 1992). At 10 mM Ca^{2+}, which is the normal extracellular concentration of Ca^{2+} in jellyfish, there was a 31 % blockade of sodium current at 0 mV (Fig. 2-3). Ca^{2+} blockade of sodium current under normal physiological

conditions presumably occurs in many marine invertebrates. Despite extreme differences in the extracellular fluid concentrations of Ca^{2+} in marine (10 mM) and terrestrial (1-2 mM) animals (Hille 1992), the relative affinity of sodium channels for Ca^{2+} appears to remain the same. Unlike Ba^{2+}, Ca^{2+} caused a significant depolarizing shift (approximately 20 mV) in the current-voltage relationship (Fig. 2-3A). One hypotehsis to explain this phenomenon is that Ca^{2+} binds with higher affinity to a region close to the voltage sensor than does Ba^{2+} or Mg^{2+}. Under the experimental conditions used here (constant concentration of divalent cations), this current / voltage shift cannot be explained by charge screening. (Worley III *et al.*, 1992) have shown that Ca^{2+} binds to the channel itself and does not interact with the surrounding lipid. The presence of Ca^{2+} binding sites on sodium channels may be an evolutionary marker of the common ancestry of calcium and sodium channels.

Effectiveness of sodium channel agonists / antagonists

Although the local anaesthetics lidocaine and procainamide produced partial blockade of sodium current in *Polyorchis penicillatus* neurons, there was no evidence of the use-dependent blockade typical of the mode of action of these drugs in most sodium channels (Hille, 1977). This result contrasts with the finding that the sodium current in *Cyanea capillata* motor neurons shows use-dependent blockade by lidocaine (Anderson,

1987). Similarly, veratridine, which modifies inactivation in most sodium channels (Hille, 1992), produced partial blockade, but did not affect inactivation kinetics either in *Polyorchis penicillatus* neurons (this study) or in *Cyanea capillata* motor neurons (Anderson, 1987). Batrachotoxin, alpha-scorpion venom and sea anemone toxin, which are also modifiers of inactivation, were also ineffective when applied to *Cyanea capillata* neurons (Anderson, 1987). A *Conus* toxin, μO-conotoxin MrVIA, which, although being a sodium channel ligand has structural similarities to the calcium channel blocking ω- and δ-conotoxins; (McIntosh *et al.*, 1995), was without effect. In contrast, μO-conotoxin MrVIA blocks TTX-insensitive sodium channels in *Aplysia californica* neurons at 350 nM (McIntosh *et al.*, 1995).

The only pharmacological agent shown to have a relatively potent blocking effect on the sodium current was capsaicin (Fig. 2-6A); however, the effect was non-specific as outward currents were also affected (Fig. 2-6B). Capsaicin has been reported to block both sodium and potassium channels in dorsal root ganglion neurons of guinea pig and chicken. Despite its lipophilicity, it was shown to act from the outside (Petersen *et al.*, 1987). Our own unpublished data also indicate that capsaicin has no effect when applied intracellularly to *Polyorchis penicillatus* motor neurons.

Effectiveness of calcium channel agonists / antagonists

The calcium channel antagonists (dihydropyridines, verapamil, diltiazem) were not effective blockers of sodium current (Figs. 2-4, 2-5) in the concentration range (20 nM to 50 μM) that is normally effective for L-type calcium channels (Hille, 1992). At higher concentrations, these antagonists are known to lose their specificity for calcium channels and begin to block sodium channels (Hille, 1992). Even at the high concentrations used here, of all the dihydropyridines examined, only nicardipine, (+)Bay K 8644 and nitrendipine showed any blocking effect (Fig. 2-4). The related compounds nimodipine and nifedipine showed no blocking effect (Fig. 2-4). On the basis of the blocking action of nicardipine and verapamil on the sodium current in neurons of the subumbrellar motor network of *Cyanea capillata*, (Anderson, 1989) suggested that the sodium channel responsible had the pharmacology of a calcium channel.

Drugs known to be calmodulin inhibitors, W7 and calmidazolium, have been shown to inhibit calcium currents (Caulfield *et al.*, 1991) and sodium currents (Ichikawa *et al.*, 1991) by directly acting upon channels (Hille 1992). Neither of these drugs had substantial blocking effects on *Polyorchis penicillatus* sodium currents (Fig. 2-5).

Because the majority of dihydropyridines and other specific antagonists of L-type calcium channels were ineffective blockers of the *Polyorchis penicillatus* sodium

channel, we are obliged to conclude that the pharmacological profile of this channel has little similarity to the profile expected for L-type calcium channels. Given that Na^+ channels appear to resemble T-type Ca^{2+} channels in structure (fig. 5-2) more than L or non-L type Ca^{2+} channels, the lack of sensitivity to L-type Ca^{2+} channel antagonists is consistent with this closer relationship with Na^+ channels with T-Type Ca^{2+} channels. Since it has been shown that a sodium channel can become Ca^{2+}-selective after the mutation of one amino acid in two domains of the pore region (Heinemann *et al.*, 1992b), it is attractive to suppose that hybrid Ca^{2+}-Na^+ channels might be present in lower metazoans. Nevertheless, despite the unusual sodium pharmacological profile of this *Polyorchis penicillatus* motor neuronal channel, it is not clear that this particular channel is more Ca^{2+}-like than other Na^+ channels.

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CHAPTER 3 A CARDIAC-LIKE SODIUM CURRENT IN MOTOR NEURONS OF A JELLYFISH[3,4]

INTRODUCTION

In hydrozoans, such as the jellyfish *Polyorchis penicillatus* every cell in the animal, except perhaps interstitial cells, are capable of generating action potentials. Because the functional roles of these excitable cells are very different, one can expect to find that the excitability properties of the cells are also diverse. The repertoire of action potential shapes, firing frequencies and firing patterns is very rich (Satterlie and Spencer, 1987). Some of this richness in excitability properties presumably is provided by suites of voltage-gated potassium currents that are known to have diverse electrical and kinetic properties (Rudy, 1988). This physiological and functional diversity of potassium channels is provided by four subfamilies of genes, *Shaker*, *Shal*, *Shab* and *Shaw* belonging to the *Shaker* family as well as other potassium channel gene families (Chandy and Gutman, 1995). It has been shown recently that the *Shaker* gene family is present in *Polyorchis* tissues (Jegla *et al.*, 1995). Although, at first glance, sodium channel proteins appear to be functionally conserved, researchers have recognized some degree of

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[3] A version of this chapter has been published. Grigoriev, N.G., Spafford, J.D., Przysiezniak, J. and Spencer, A.N. (1996) *Journal of Neurophysiology*. 76(4):2240-2249.
[4] This chapter was a collaborative effort between Grigoriev and Spafford.

physiological and structural diversity (Goldin, 1995). For example TTX-sensitive and TTX-resistant channels can be clearly distinguished by a wide range of electrophysiological and pharmacological characteristics (Yoshida, 1994). In this study we examined the electrophysiological properties of a TTX-insensitive sodium current in swimming motor neurons (SMNs) of *P. penicillatus*. We describe a number of electrophysiological features of this current that are "cardiac-like" rather than "neuron-like". In contrast, a second neuronal type, the B neuron, has properties that are more similar to most neuronal sodium currents. This divergence of physiological properties is discussed with respect to the functional roles of the cells involved.

MATERIALS AND METHODS

Cell culture

Medium-sized jellyfish (diameter 20-30 mm) were collected in Bamfield and Grappler Inlets (Vancouver Island, British Columbia) and maintained at approximately 10° C in flow-through aquaria. Cells were cultured using a method from (Przysiezniak and Spencer, 1989) with slight modifications. Cells were exposed to collagenase for a shorter period (1 hr instead of 4-5 hr), with agitation. Recordings were made using cell cultures that were no more than three days old.

Swimming motor neurons (SMNs) were identified by their large size (soma 30-50 μm long, with processes up to 200 μm), clear cytoplasm and a nucleus surrounded by membranous structures. Typically, isolated motor neurons were fusiform or lamelliform but lacked dendrites. Neurons ranged in length from 80-200 μm and had a mean capacitance of 41.3 pF (n = 21). SMNs have a characteristic action potential that is identifiable *in vitro* and *in vivo*. Major features of the sodium currents, such as time to peak, peak current, voltage-dependence, time course of responses, did not change with the age of culture.

Whole-cell recordings

Whole cell, tight-seal recordings were made using electrodes of borosilicate glass tubing (TW-150-4, World Precision Instruments), with resistances of 1-2 MΩ when filled with electrode solutions. Recordings were made at room temperature with an Axopatch-1D amplifier (Axon Instruments), low-pass filtered at 3 kHz using a 4-pole Bessel filter, and digitized using a Labmaster TL-125 acquisition board (Axon Instruments).

Cultures were viewed under phase contrast with a Nikon Diaphot inverted microscope. Fine alignments of the micro-electrode near the cell surface were made with a Peizo-electric driver (Burleigh). Stimulus control, data acquisition and analysis were performed with pCLAMP 6.0 software (Axon Instruments) on an IBM-PC 486 computer.

Leakage and capacitive currents were subtracted using -P/4 or -P/5 protocols, from a holding potential of -80 mV, before test pulses eliciting active responses were applied. Series resistances (R_s) were compensated optimally to minimize voltage errors (R_s compensation was usually set to values of 80% or more). C_m (membrane capacitance) and R_s values were obtained by minimizing the capacitive transient in response to a hyperpolarizing voltage step. Only recordings that met the following criteria were used in the analysis: 1) Peak uncompensated current was > 2 nA; R_s was 2 - 4 MΩ before compensating for R_s. 2) C_m and R_s did not change by more than 10% during experiments. 3) Leakage resistances were in the range of 100 MΩ to 0.5 GΩ. 4) After series resistance compensation, sodium currents reached their peak within 800 μs using test pulses with voltage steps from -80 mV to +10 mV.

All experiments were performed at room temperatures (20°C - 22°C), which were close to the surface water temperatures at the collection site (17°C – 20°C).

Lithium, guanidinium, and rubidium solutions were micro-perfused in bath solution dispensed from a manifold with a dead volume of less than 1 μl. Flow, by gravity, was controlled by electro-mechanical pinch valves and the rate of flow provided complete solution exchange in 2 s. As soon as a current tracing superimposed on the previous trace, this was recorded as the experimental trace (saturation) and washing was started. The maximal effect was obtained from 30 s to 2 min after application of monovalent cations.

Solutions

Before use, all culture and recording solutions were filtered through cellulose acetate membrane cartridges with 0.2 μm pore-size. Artificial seawater, which contained (in mM) 376 NaCl, 26 $Na(SO_4)_2$, 41.4 $MgCl_2$, 10 $CaCl_2$, 8.5 KCl, 10 *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-hemisodium salt, gentamycin sulphate 50 mg/ml, was titrated to pH 7.5 with HCl. Electrode solutions, which contained (in mM) 50 NaCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES-free acid, 11 ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 350 CsCl, 30 CsOH and 20 TEA-Cl, were titrated to pH 7.5 with HCl. Standard bath solutions were calcium-free. contained (in mM) 395 NaCl, 50 $MgCl_2$, 30 *N*-methyl glucamine-HCl, 10 HEPES-sodium salt, and titrated to pH 7.5 with HCl. All outward currents were totally blocked within 5 min after break-through, and inward calcium currents were eliminated since the external solution was calcium-free.

Data analysis

In Figs. 2, 3, 6 and 7, data points from all selected cells were averaged and curves fitted to the means using the relevant functions. Thus the fitted curves could have slightly different parameters than the mean values given in Table 3-1, where the deduced parameters were averaged after curve fitting.

RESULTS

Methods used for isolating sodium current

Sodium currents were isolated by blocking potassium currents with intracellular administration of Cs^{2+} and tetraethylammonium (TEA) and by eliminating calcium currents by using a Ca^{2+}-free extracellular solution. The efficiency of this method for isolating sodium currents was checked by using a sodium-free extracellular solution when potassium and calcium currents were eliminated. Under these conditions, we have not recorded any membrane currents in the voltage ranges used in this study. We cannot exclude the possibility that a small component of the "sodium" currents recorded were in fact carried by sodium ions permeating potassium channels. However, kinetic properties of potassium currents in these neurons (Przysiezniak and Spencer, 1994) are quite different from the kinetics of the sodium currents recorded in this study. Thus, if present, sodium currents that permeated potassium channels must have been very small.

Table 3-1 summarizes the parameters and properties of the sodium current recorded from swimming motor neurons of the hydromedusan *Polyorchis penicillatus*.

Table 3-1

Quantitative summary of parameters of sodium current in swimming motor neurons of *Polyorchis penicllatus*

Parameter	mean ± S.E.M.	n
I_{max}[a]	7.97 ± 0.15 nA	22
$I_{density}$[a,b]	0.20 ± 0.03 mA/cm^2	22
Electrical properties[c]		
Va[c]	-19.68 ± 0.42 mV	8
Ka[c]	-5.81 ± 0.09 mV	8
V_i[d]	-29.18 ± 0.53 mV	16
Ki[d]	-4.40 ± 0.12 mV	16
Kinetical properties		
Activation (time to peak)[e]		
Time to I_{peak} [a]	0.74 ± 0.06 ms	14
B_1	7.36 ± 0.15 mV	14
B_2	147.1 ± 4.34 mV	14
A_1 / A_2	3.97 ± 0.04	14
Inactivation		
Inactivation decay[a,f]		
τ_1 (fast)	1.91 ± 0.07 ms	16
τ_2 (slow)	11.65 ± 0.55 ms	16
A_1 / A_2	2.02 ± 0.04	16
τ_3 (very slow)	241 ± 15.23 ms	3
Steady-state inactivation for two components of decay[g]		
$V\tau_1$ (fast)	-31.17 ± 0.44 mV	16
$K\tau_1$	-4.49 ± 0.14 mV	16
$V\tau_2$ (slow)	-24.26 ± 0.39 mV	16
$K\tau_2$	-4.98 ± 0.29 mV	16
Recovery from inactivation[h]		
τ_1	66.67 ± 0.76 ms	8
$\tau 2$	113.7 ± 3.43 ms	8
A_1 / A_2	4.47 ± 0.08	8
time of half recovery ($t_{1/2}$)	52.6 ± 2.9 ms	8
time of complete recovery	500 ± 38 ms	8

Footnotes for Table 3-1 (shown on previous page)

[a]Parameters calculated from maximal current generated from a voltage step to +10 mV from -80 mV.

[b]Calculated from whole-cell capacitance (41.33 +/- 0.03 pF, n=22) assuming 1 $\mu F/cm^2$. Capacitance was read from dials after minimizing capacitive current on patch clamp amplifier.

[c]Peak current data for each cell were fitted with a combination of a Boltzmann equation and Ohm's law: $I = G_{max} \times (V_c - E_r) / \{1+\exp[(V_a - V_c)/K_a]\}$, where V_c is the command voltage, G_{max} is the maximal sodium conductance, E_r is the fitted reversal potential of the current, V_a is the voltage of half-activation, and K_a is the slope factor of the activation curve in millivolts per *e*-fold change.

[d]For steady-state inactivation data, each cell was fitted with a Boltzmann equation: $I_{test} = I_{max} /\{1+\exp[(V_{pp}-V_i)/K_i]\}$, where I_{test} is the peak current amplitude expected on the test pulse response, I_{max} is the maximal current fitted at non-inactivating prepulse voltages, V_{pp} is the prepulse voltage, V_i is the prepulse voltage causing half-inactivation, and K_i is the slope factor of the inactivation curve in mV per *e*-fold change.

[e]Time-to-peak data were fitted with two exponents: $A_1 *\exp(-V_c/B_1) + A_2*(\exp(-V_c/B_2)$, where V_c is the command voltage, B_1 and B_2 are constants of the fast and slow components, A_1 / A_2 is the relative amplitude of fast and slow components, and I / I_{max} is the normalized current. Fitting was done using the Marquardt-Levenberg algorithm.

[f]Time constants ($\tau 1$ and $\tau 2$) and relative amplitude of fast and slow components of inactivation (A_1/A_2) were found by fitting sodium current inactivation decay with two exponents: $A_1 *\exp(-T/\tau_1) + A_2*(\exp(-T/\tau_2)$, where T is time and I / I_{max} is the normalized current. Fitting was done using the Marquardt-Levenberg algorithm.

[g]Cell recordings were fitted with two exponentials (same as in (e) but V_{pp} replaces V_c) using the Chebyshev method. Each exponent was normalized to maximal currents and fitted with a Boltzmann equation (same as in (d) but $V\tau_1$ or $V\tau_2$ replaces V_i and $K\tau_1$ replaces K_i).

[h]Recovery from inactivation data was normalized to maximal currents (I/I_{max}) and curve fitted with two exponents: $I / I_{max} = 1- \{A_1 *\exp(-T/\tau_1) + A_2 *\exp(-T/\tau_2)\}$, where T is time.

Sodium-selective inward current

Figure 3-1A shows a representative family of rapidly activating and inactivating sodium currents generated in conditions where potassium and calcium currents were eliminated. Maximal sodium current was produced in response to test pulses of + 10 mV from a holding potential of - 80 mV. In this typical experiment the sodium current reversed at +56 mV (Fig. 3-1C), which was close to the Nernst potential for sodium (+ 52 mV). In conditions where there was no external sodium and the extracellular calcium concentration was 100 mM, a small, slower activating and inactivating inward current was seen (Fig. 3-1B) in addition to a family of very rapidly activating and inactivating outward sodium-dependent currents (Fig. 3-1B and 1C). It should be noted that the kinetics of the inward calcium current are very different from the sodium current and measurement of the peak outward sodium current (Fig. 3-1B) does not indicate any inward components, as shown in Fig. 3-1C. This show that these channels are highly selective for sodium ions.

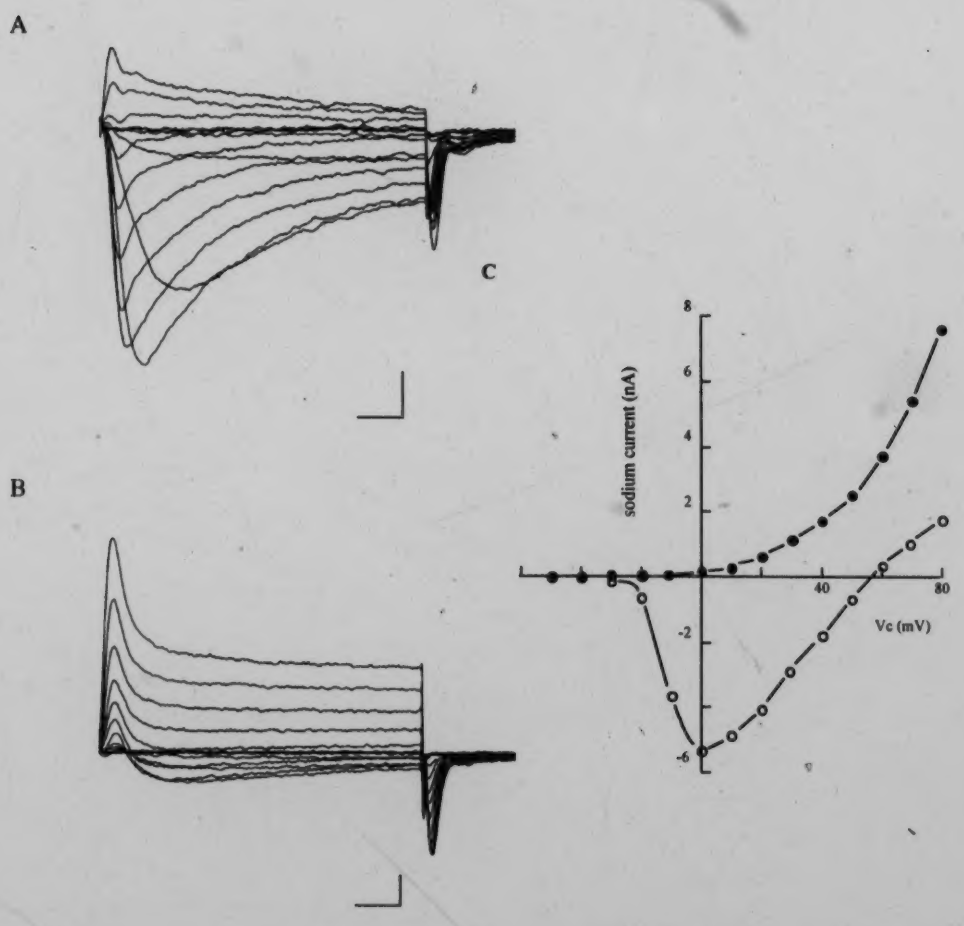

Figure 3-1. Sodium selectivity and voltage dependence of sodium current in swimming motor neurons of *P. penicillatus*. Potassium currents were blocked by using an electrode solution containing Cs^{2+} and tetraethylammonium (TEA). A) Representative sodium current responses observed using a calcium-free, sodium-containing bath solution with (in mM) 395 NaCl, 50 $MgCl_2$, 30 *N*-methyl glucamine-HCl, and 10 *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) sodium salt and titrated to pH 7.5 with HCl. Test pulses lasting 10 ms were applied from a holding potential of -80 mV, to a series of command voltages (Vc) incremented from -50 to +80 mV in steps of +10 mV. B) Outward sodium current and a slowly activating and inactivating inward calcium current observed using a sodium-free, calcium-containing bath solution with (in mM): 100 $CaCl_2$, 50 $MgCl_2$, 225 *N*-methyl glucamine-HCl, 10 HEPES-sodium salt and titrated to pH 7.5 with HCl. Voltage-clamp protocols as for A. Vertical and horizontal bars for both A and B are 1 nA and 1 ms, respectively. C) Current-voltage relationships of the peak sodium currents in A (○) and B (●).

Permeability to monovalent cations

The ionic permeability of the channel population, responsible for the sodium current, to lithium, rubidium and guanidinium was measured. These channels were almost as permeable to lithium as sodium ($P_{Li}/P_{Na} = 0.941$), but they were impermeable to rubidium, whereas guanidinium was slightly permeable ($P_{guanidinium}/P_{Na} = 0.124$; see Table 3-2).

Electrical properties

Figure 3-2 shows the conductance / voltage and steady-state inactivation curves for sodium current. Activation was first detected at approximately - 40 mV and was maximal at + 10 mV. At - 15 mV, the current was almost completely inactivated and became fully available at - 50 mV.

Table 3-2

Ionic permeabilities of sodium current in swimming motor neurons of *Polyorchis penicillatus* versus sodium currents in other species[a]

	P_X / P_{Na}			
Monovalent ion	*Polyorchis* SMN	Frog node[b]	Squid axon[c]	*Myxicola* axon[d]
lithium	0.941	0.93	1.1	0.94
guanidinium	0.124	0.13	0.178	0.17
rubidium	<0.001	<.012	0.025	

[a]Relative permeabilities of sodium channels to the test ion (P_x / P_{Na}) were calculated from the change in reversal potential (E_r) by measurement of peak currents as shown in Fig. 3-1C using voltage steps to +80 in 10 mV increments from -40 mV. Holding potential was -80 mV. Changes in E_r in lithium and guanidinium were -1.0 mV and -53.2 mV, respectively. There was no current reversal in rubidium. Change in E_r on substitution of sodium with a monovalent ion (X): $E_r(x) - E_r(Na) = 58.17 \log_{10} P_x/P_{Na} (X) / (Na)$, where parentheses refer to ionic activities in the external solution, and reversal potentials are in mV. LiCl, guanidinum chloride and RbCl (395 mM) were used in place of NaCl in the standard bath solution which contained (in mM) 395 NaCl, 50 $MgCl_2$, 30 *N*-methyl glucamine-HCl, and 10 *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) sodium salt and titrated to pH 7.5 with HCl.

[b](Hille, 1971)

[c](Chandler and Meves, 1965)

[d](Binstock, 1976), (Ebert and Goldman, 1976)

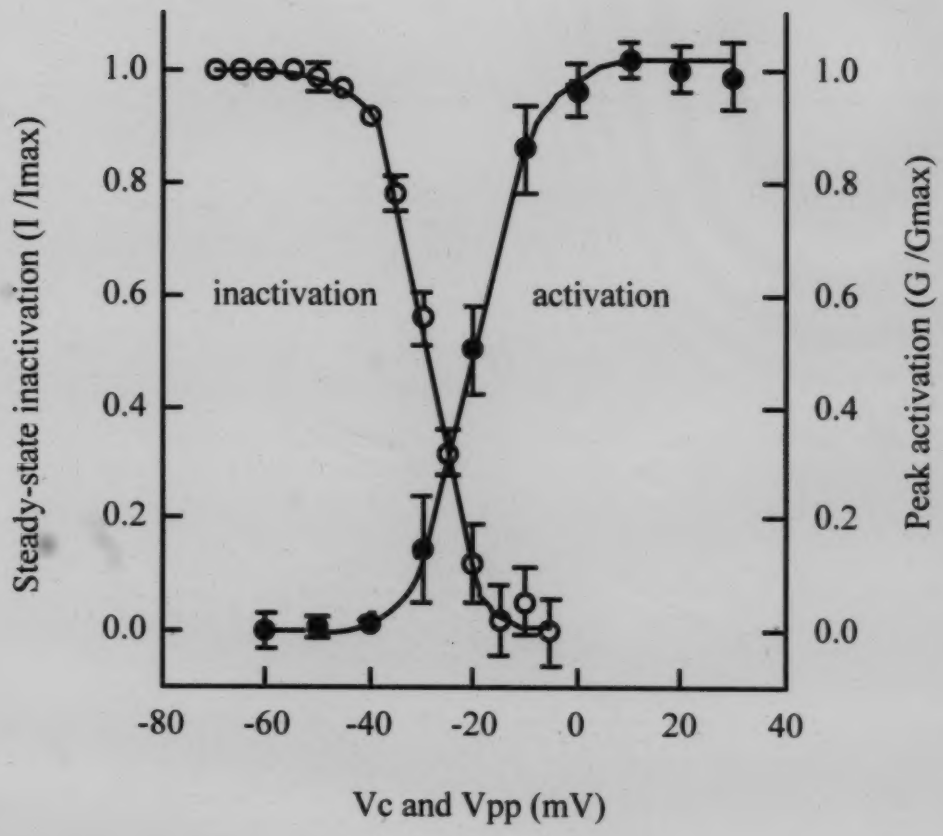

Figure 3-2. Activation (●) and steady-state inactivation curves (○) for of sodium current in swimming motor neurons of *P. penicillatus*. Activation curve was generated using test pulses lasting 30 ms, applied from a holding potential of -80 mV, to a series of command voltages (V_c) incremented from –60 to +30 mV in steps of +10 mV. Activation data were normalized to maximal conductance (G / G_{max}), then averaged and fitted to the means with a combination of a Boltzmann equation and Ohm's law (Table 3-1, equation in footnote c). Inactivation curve was generated using 20 ms test pulses to +10 mV following 2 s inactivating prepulses (V_{pp}) from -70 mV to +5 mV in 5 mV increments. Inactivation data were normalized to maximal current (I / I_{max}), averaged and fitted to means with a Boltzmann equation (Table 3-1, equation in footnote d). n = 8 cells for activation and 16 for inactivation, error bars are S.E.

Kinetical properties

The rate of activation of the sodium current was steeply voltage-dependent (Fig. 3-3) and could be fitted with two exponents: (7.36 +/- 0.15 mV) and (147.1 +/- 4.34 mV). Time to peak at maximal current was 0.74 +/- 0.06 ms. For all cells, the time course of inactivation could be fitted with two exponents, fast (τ_1 = 1.91 +/- 0.17 ms at + 10 mV) and slow (τ_2 = 11.65 +/- 1.05 ms at + 10 mV). Most neurons (97.8 %) showed inactivation kinetics that were almost identical (Fig. 3-4A). On the basis of fitted inactivation data from 16 such cells, we obtained a ratio of 2.02 +/- 0.082 between the amplitudes of the fast and slow inactivation components. However, two neurons showed time courses of inactivation in which the fast component was dominant (Fig. 3-4B), and three neurons showed inactivation where only the slow component is present (Fig. 3-4C). Using 2-s pulses (data not shown) to - 20 mV, it was possible to see an additional, very slowly inactivating sodium current with an inactivation time constant of 241 +/- 15.23 ms.

The voltage-sensitivities of fast and slow components of inactivation were examined (Fig. 3-5). Both exponents showed similar voltage-dependence. The two components τ_1 and τ_2 exhibit different voltage sensitivities of steady-state inactivation (Fig. 3-6) with the curve for τ_2 shifted to the right along the voltage axis relative to τ_1 by 6.91 mV.

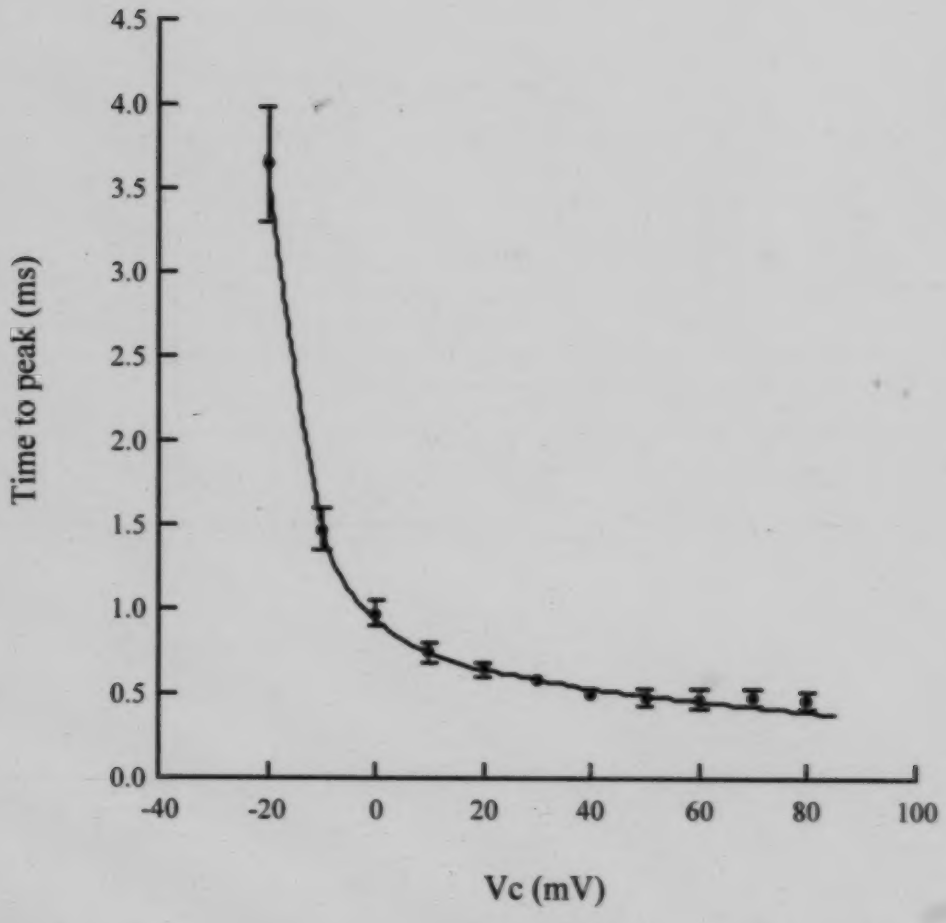

Figure 3-3. Voltage-dependence of time to peak of sodium current in swimming motor neurons of *P. penicillatus*. Time-to-peak data were collected using test pulses lasting 10 ms, applied from a holding potential of -80 mV, to a series of command voltages (V_c) incremented from –20 mV to +80 mV in steps of +10 mV. Time-to-peak data were averaged and fitted to means with a double-exponential function (Table 3-1, equation in footnote e). n = 14 cells, error bars are S.E.

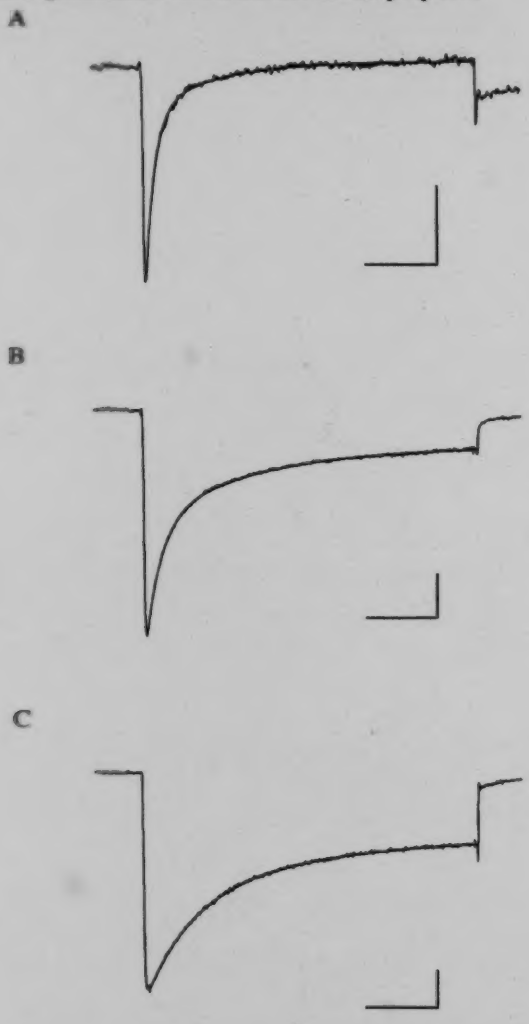

Figure 3-4. Variation in inactivation kinetics of sodium current in swimming motor neurons (SMNs) of *P. penicillatus*. A) Representative sodium current trace from an SMN where the fast component of inactivation predominates. τ_1 was calculated to be 1.76 ms and τ_2 was 10.48 ms. Ratio of amplitudes of the component currents (A_1/A_2) showing fast and slow inactivation was 3.16. B) Representative sodium current trace from a SMN showing a combination of fast (τ_1) and slow (τ_2) inactivation. τ_1 was calculated to be 2.18 ms and τ_2 was 11.94 ms. Ratio of the amplitudes of component currents (A_1/A_2) showing fast and slow inactivation was 1.94. For A and B, inactivation curve was fitted with a double exponential function: $A_1*\exp(-10/\tau_1) + A_2*(\exp(-10/\tau_2)$, where τ_1 (fast) and τ_2 (slow) are time constants and A_1/A_2 are amplitudes of component currents showing fast and slow inactivation. C) Representative sodium current trace from a SMN where only slow component of inactivation, τ_2 = 10.67 ms, is present. Inactivation curve was fitted with a single exponential function: $A*(\exp(-10/\tau)$. In A,B and C, 50 ms test pulses to + 10 mV were given from a holding potential of -80 mV. Vertical and horizontal bars are 1 nA and 10 ms.

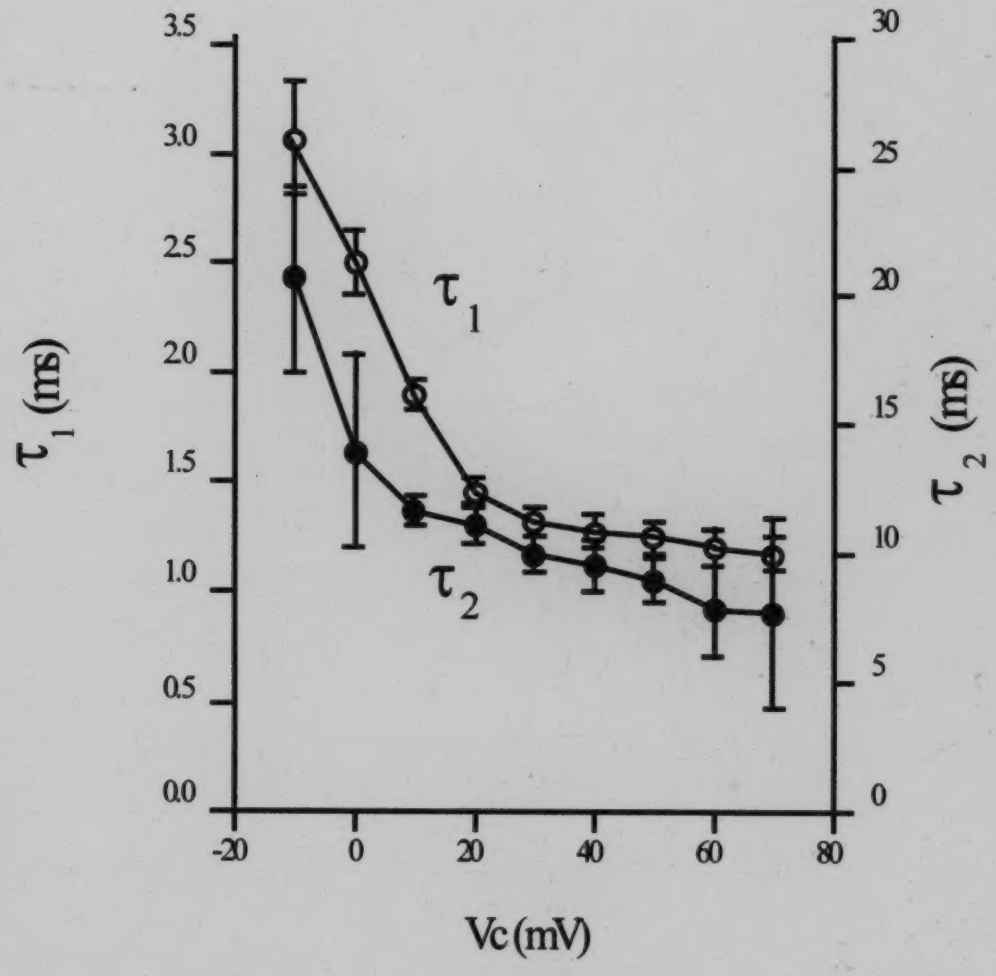

Figure 3-5. Voltage-dependence of fast and slow components of inactivation of of sodium current in swimming motor neurons of *P. penicillatus*. Fast (τ_1, O, left ordinate) and slow (τ_2, ●, right ordinate) time constants of inactivation are plotted against the command voltage (V_c). Voltage-clamp protocols and raw current recordings were as for Fig. 3-2. Time constants were calculated using a double exponential function (Table 3-1, equation in footnote f). n = 14 cells, error bars are S.E.

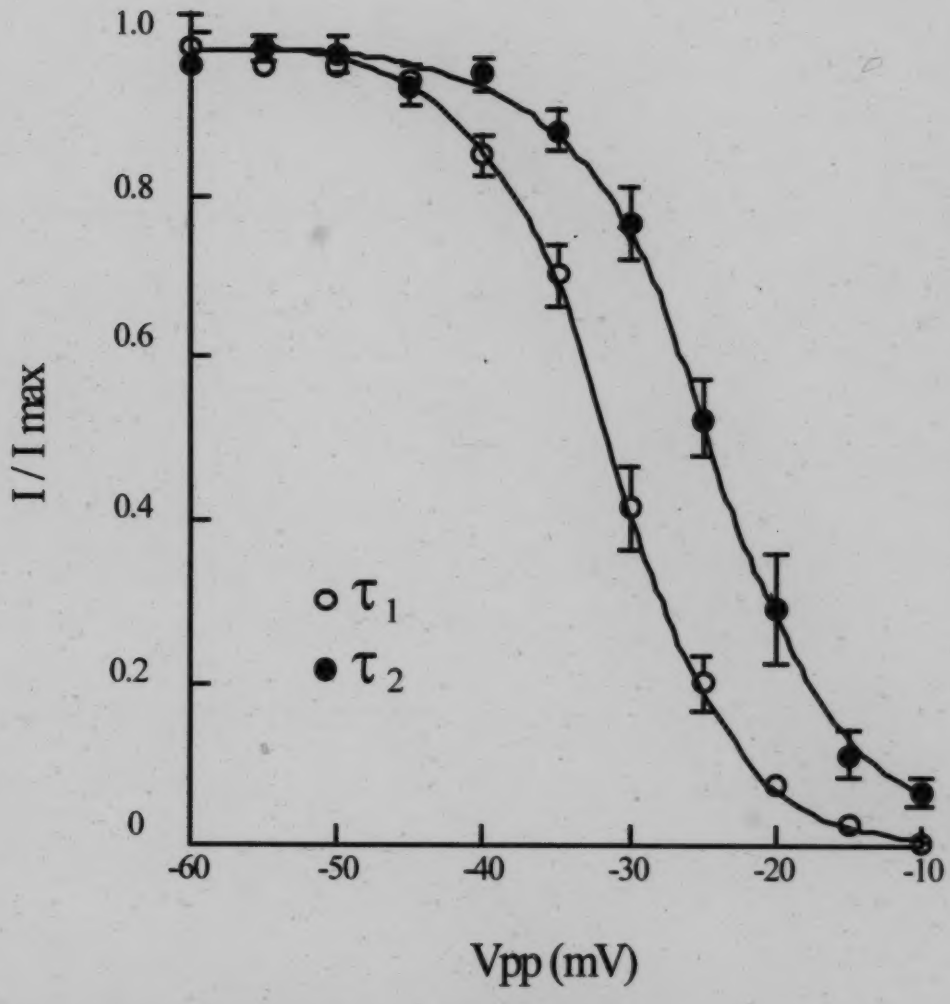

Figure 3-6. Steady-state availability of fast (τ_1, ○) and slow (τ_2, ●) components of inactivation as a function of prepulse voltage (V_{pp}) of sodium current in swimming motor neurons of *P. penicillatus*. Voltage-clamp protocols and raw current recordings were as for Fig. 3-2. Time constants were calculated using a double exponential function (Table 3-1, equation in footnote f). The current amplitudes of fast and slow inactivation components were normalized to maximal current (I/ I_{max}) then averaged and fitted to the means with a Boltzmann equation (Table 3-1, equation in footnote g). n = 14 cells, error bars are S.E.

Two distinct time constants of recovery from inactivation were apparent, 66.67 +/- 0.76 ms and 113.7 +/- 3.43 ms with complete recovery occurring within 500 +/- 38 ms, and with a time to half recovery of 52.6 +/- 4.9 ms (Fig. 3-7).

A neuronal class with rapid recovery from inactivation

Another subset of central neurons, B neurons, have distinct morphologies and excitability properties to SMNs in mixed cultures (Przysiezniak and Spencer, 1989). *In vivo* these neurons produce short-duration spikes that commonly occur in bursts (Spencer and Arkett, 1984). Using the same experimental conditions as for recordings of SMNs, sodium currents were recorded from five of these B neurons (Fig. 3-8). Inactivation of sodium current in B neurons could be fitted with two exponents, with the fast component, τ_1, being similar to SMNs (approximately 2 ms) but the slow component, τ_2, having a much longer time constant of 88 ms (cf. 12 ms for SMNs). The sodium current recorded from B neurons showed far more rapid recovery from inactivation (half recovery < 5 ms). Figure 3-9A shows one such recording together with a comparable recording from a SMN (Fig. 3-9B). Differences in the time courses of recovery from inactivation also are shown graphically (Fig. 3-9C).

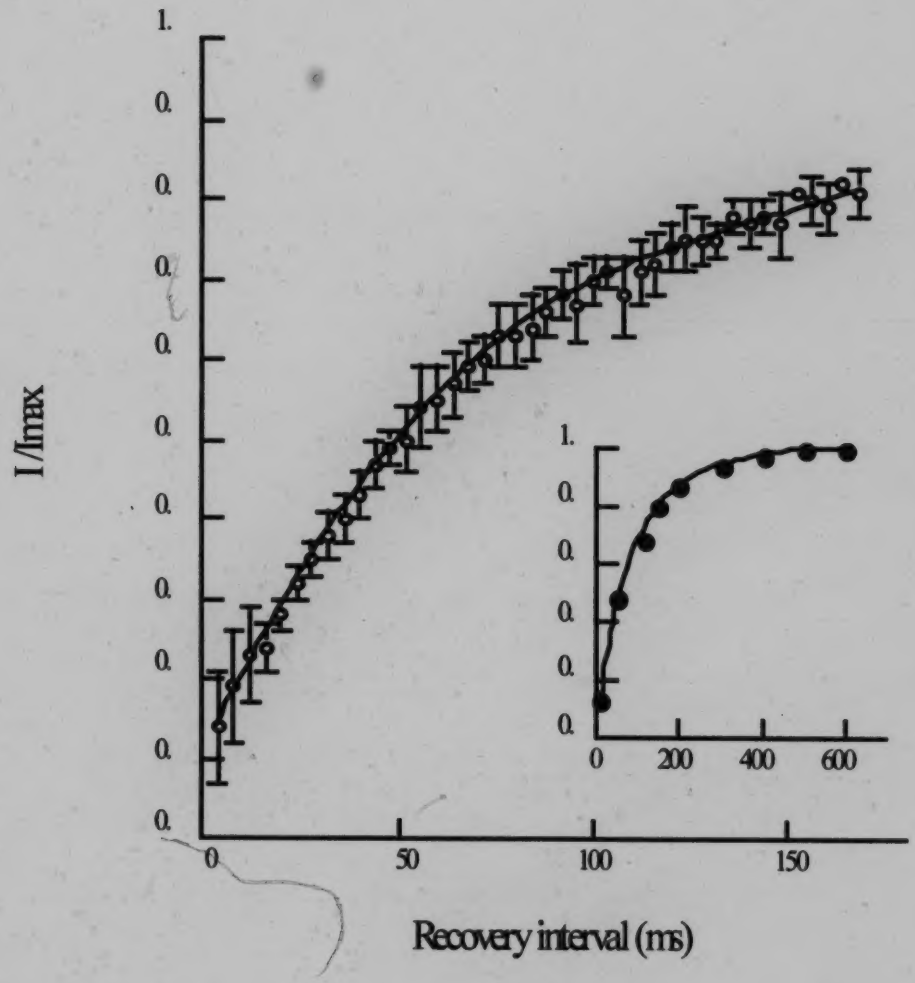

Figure 3-7. Time course of recovery from inactivation of sodium current in swimming motor neurons of *P. penicillatus*. Recovery from inactivation data were collected using a two-pulse protocol composed of a 100 ms inactivating prepulse applied from a holding potential of -80 mV to +10 mV, followed by a recovery period of variable duration at -80 mV and a test pulse to +10 mV, lasting 20 ms. Peak currents obtained in response to test pulses were normalized to prepulse values (I / I_{max}) then averaged and fitted to means with two exponents (Table 3-1, equation in footnote h). n = 8 cells, error bars are S.E. *Inset*: Graph using different time scale to show complete recovery from inactivation. n = 8 cells, error bars are not shown as they are smaller than symbols.

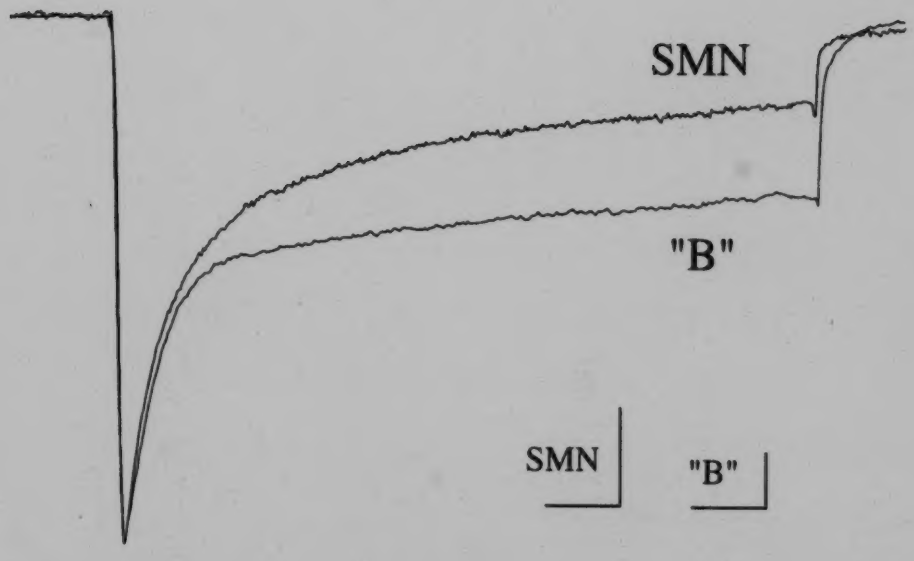

Figure 3-8. Sodium currents in swimming motor neurons (SMNs) and B neurons of *P. penicillatus* show different kinetics of slow inactivation. Superimposed, representative current traces from a SMN (*top*) and a B neuron (*bottom*) neuron using 50 ms test pulses to + 10 mV from a holding potential of -80 mV stepped to +10 mV. Vertical and horizontal bars are 1 nA and 5 ms, respectively.

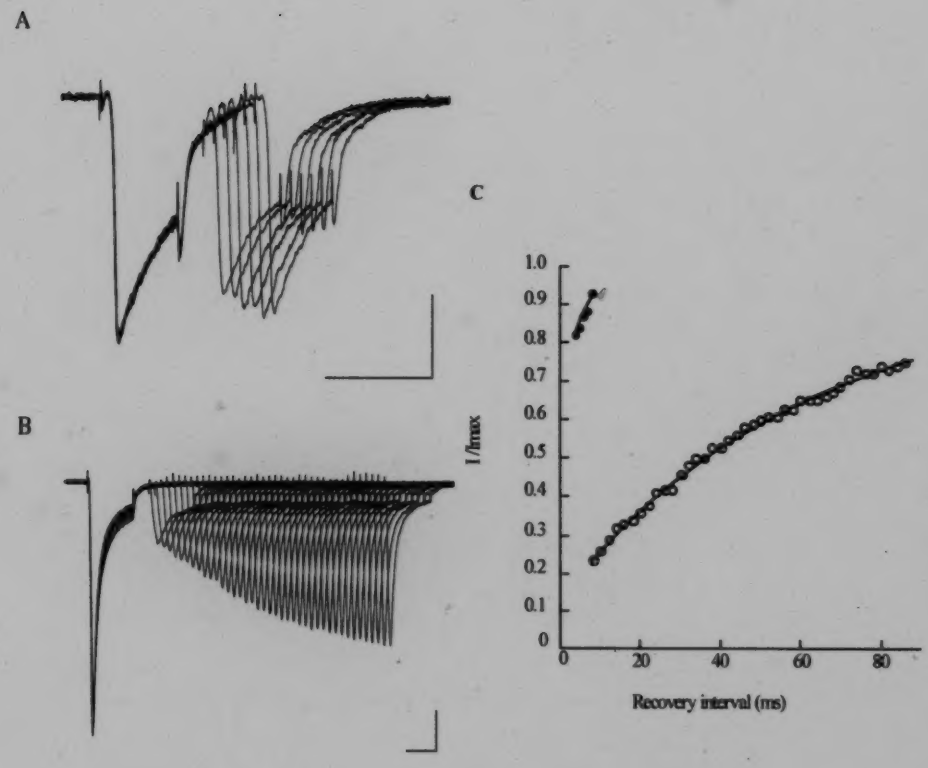

Figure 3-9. Comparison of time courses of recovery from inactivation of sodium currents in swimming motor neurons and B neurons of *P. penicillatus*. A) Current traces showing recovery from inactivation of a typical B neuron. A two-pulse protocol was used consisting of a 10 ms, inactivating prepulse applied from a holding potential of -80 mV to +10 mV, a recovery period at -80 mV of variable duration from 4 to 8 ms and a 10 ms test pulse to +10 mV. B) Current traces showing recovery from inactivation of a typical SMN. A two-pulse protocol was used consisting of a 20 ms, inactivating prepulse applied from a holding potential of -80 mV to +10 mV, a recovery period at -80 mV of variable duration from 8 to 86 ms and a 20 ms test pulse to +10 mV. C) Recovery from inactivation shown graphically for B neurons (●) and SMN (○). Peak currents obtained in response to test pulses were normalized to prepulse values (I / I_{max}) and fitted with two exponents (Table 3-1, equation in footnote h). Vertical and horizontal bars are 1 nA and 5 ms, respectively.

DISCUSSION

Sodium is the major carrier of current for generation of action potentials

The sodium current that we describe is undoubtedly the major inward current responsible for generating action potentials in swimming motor neurons. Anderson, (1979) showed that bathing this motor network in a sodium-free solution abolished propagated events but that a calcium-free solution supported continuous, bursting activity. Nevertheless, at least two high-voltage activated (HVA-t and HVA-s) and a possible low-voltage activated calcium current, have been described from these motor neurons in addition to a rapid sodium current (Przysiezniak and Spencer, 1992). It is important to note that the peak current carried by calcium (0.22 nA) (Przysiezniak and Spencer, 1992), under normal ionic conditions, was far less than that carried by sodium (8.0 nA, this study) and is therefore unlikely to support calcium-dependent action potentials. We were able to confirm that sodium- and calcium-dependent currents are separable and pass through different channel populations (Fig. 3-1B), although it is not clear whether a proportion of the inward calcium current we recorded diffuses through sodium-selective channels (Meves & Vogel 1973). Sodium and calcium currents are distinguishable by their kinetics, with calcium currents being far slower (Table 3-1 in (Przysiezniak and Spencer, 1992). Further separation of sodium- and calcium-dependent currents is possible when pharmacological data is considered. Przysiezniak and Spencer

(1992) reported that the HVA-t calcium current was blocked almost completely by 0.3 mM Cd^{2+} whereas 1 mM Cd^{2+} only blocked 45% of the sodium current (see Chapter 2), and 100 μM nifedipine blocked HVA-s, yet the sodium current is insensitive to nifedipine (see Chapter 2).

A noticeable feature of the action potentials of SMNs is that they have a variably expressed plateau phase that has been shown to modulate transmitter release onto the post-synaptic, epithelio-muscular cells (Spencer, 1981; Spencer *et al.*, 1989). Because the sustained calcium current, HVA-s, is almost two orders of magnitude smaller than the very slowly inactivating component of the sodium current, we can be fairly certain that the plateau of SMN spikes is maintained mostly by sodium influx. It was previously shown that the duration of the plateau is determined by the relative amplitude of a rapidly inactivating potassium current $I_{K\text{-fast}}$, which becomes more available after a period of hyperpolarization (Przysiezniak and Spencer, 1994; Spencer *et al.*, 1989).

Similarity of Polyorchis motor neuronal sodium currents to other sodium currents

As shown in Table 3-2, lithium, rubidium and guanidinium have very similar relative permeabilities through these *Polyorchis* sodium channels when compared with sodium channels in other species. This similarity presumably reflects a conserved pore size that provides ionic selectivity (Hille, 1992).

Although the slopes of the activation and steady-state inactivation curves of the SMN sodium current are similar to those recorded for other sodium currents, the voltages for 50% activation and steady-state inactivation (-19.7 mV and -29.2 mV, respectively) are shifted some 40 mV in a depolarizing direction relative to many TTX-sensitive sodium currents (eg. squid giant axon (Hille, 1992) and dorsal root ganglionic neurons (Roy and Narahashi, 1992)).

In comparison, TTX-resistant currents often show strong depolarizing shifts (Bossu and Feltz, 1984; Ikeda *et al.*, 1986; Roy and Narahashi, 1992). A positive shift in voltage sensitivity also was reported for the TTX-insensitive sodium current in scyphozoan motor neurons (Anderson, 1987) as well as other cnidarian calcium (Anderson, 1989; Przysiezniak and Spencer, 1992) and potassium (Anderson and McKay, 1987; Dunlap *et al.*, 1987; Holman and Anderson, 1991; Jegla *et al.*, 1995; Przysiezniak and Spencer, 1994) currents. Although such positive shifts in voltage sensitivity may be common in cnidarians, there are exceptions, such as the calcium current in giant axons of *Aglantha* (Meech and Mackie, 1993). Other lower taxa such as protozoans (Oertel *et al.*, 1977), ctenophores (Dubas *et al.*, 1988) and flatworms (Blair and Anderson, 1993) have currents with similar depolarizing shifts in their activation and steady-state inactivation curves. The apparent matching of voltage sensitivities of inward and outward currents is required presumably for generation of action potentials, though the reason why many excitable cells in lower taxa are operating in different voltage ranges is as yet unclear. The *in vivo* resting potential of the swimming motor neuron

network varies significantly from -25 mV to - 50 mV depending on the sum of synaptic activity from afferent neurons at local sites (Spencer, 1981). This has significant physiological consequences for the excitability properties of the network. For example, it has been shown that the membrane potential just before generation of action potentials in these neurons regulates the duration of the action potential (Spencer, 1981; Spencer *et al.*, 1989). It also may be functionally relevant that the steepest part of the steady-state inactivation curve for sodium current (Fig. 3-2) falls within the voltage range of the normal *in vivo* resting membrane potentials and it also might be expected that the time to peak of action potentials, as well as the plateau, is modulated via the resting membrane potential.

Activation of sodium current in *Polyorchis* motor neurons is complete in a range of 0.7 to 3.7 ms which is similar to the range (0.6 - 5.0 ms) for canine cardiac Purkinje cells (Hanck and Sheets, 1992) but slower than in many neurons such as scyphomedusan motor neurons (0.8 - 1.4 ms at 22 – 25°C) (Anderson, 1987), squid giant axon (0.4 - 1.0 ms at 10°C) (Armstrong *et al.*, 1973) and expressed mammalian brain sodium channels (0.1 - 0.2 ms at 20 - 24°C (Fleig *et al.*, 1994)).

The existence of two components of inactivation may reflect the presence of two channel populations having different inactivation time constants, two kinetic modes of inactivation in a single channel population (Fleig *et al.*, 1994), or differential expression of auxillary subunits (Isom *et al.*, 1992). The first explanation seems unlikely because the kinetics of inactivation would be the only parameter distinguishing the two channel

populations. Our data does not help us to distinguish clearly between the latter two explanations, because a rightward shift of the slow component in steady-state inactivation curves along the voltage axis relative to the fast component (Fig. 3-6) has been reported for both mechanisms (Fleig *et al.*, 1994; Isom *et al.*, 1992). However, during activation, there are no noticeable differences in the voltage sensitivities of the slow and fast components of inactivation (Fig. 3-5), which was reported when the α-subunit of rat skeletal muscle sodium channel was coexpressed with the β-subunit of human brain (Cannon *et al.*, 1993). Furthermore, expression of α-subunits alone produced macroscopic current with two time constants of inactivation, which varied more than 10 fold, and possessed noticeable shifts in activation curves (Fleig *et al.*, 1994). A five to ten-fold difference between fast and slow components of inactivation in our data (Fig. 3-5) is similar to the increase in the rate of inactivation of macroscopic sodium current reported when β-subunits were coexpressed with α-subunits (Bennett *et al.*, 1993; Cannon *et al.*, 1993; Isom *et al.*, 1992). Slow or fast components of inactivation would predominate in neurons where the proportions of β-subunits to α-subunits are different.

Similarity of properties of sodium current in SMNs with those of cardiac Purkinje cells

Swimming motor neurons (SMNs) form an electrically coupled, compressed network that is both the pacemaker and transmission route for motor spikes innervating the swimming muscle sheets, which, when they contract, jet water out of the bell opening (Spencer, 1981; Spencer, 1982). As in the heart, the spreading of excitatory waves in the SMN network leads to synchronized contractions of muscle cells with a striated pattern, tightly-coupled by gap junctions (Lin and Grigoriev, 1998). Like mammalian cardiac Purkinje fibers, there is a 1:1 relationship between motor spikes and muscle contractions. In both cases, the spreading of the excitatory wave is carried in a closed network that is prone to re-entry. Once an ectopic wave is initiated, it impairs the ordered, directional information that leads to synchronous contractions initiated by the pacemaker (Berne and Levy 1998; Fye 1987) Both the heart and bell of jellyfish form fluid pumps. Because there are similar physical constraints for effective pumping, the modes of muscular contraction are similar.

SMNs in adult jellyfish, rarely produce spiking frequencies exceeding 1 Hz, and a long refractory period ensures that swimming motor neurons do not fire prematurely. Premature excitation of the swimming muscle would be ineffective because the bell of the jellyfish would not have sufficient time to refill. The duration of the refractory period is determined mainly by the kinetics of recovery from inactivation. Like other sodium currents, such as those in rat dorsal root ganglia (Ogata and Tatebayash, 1993) and rat

pituitary melanotroph (Kehl, 1994), recovery from inactivation in *Polyorchis* motor neurons could be fitted by two exponents. Neuronal sodium currents generally show rapid recovery from inactivation with half-recovery times ($T_{1/2}$) of approximately 5 ms, as in giant axons of squid at 19° C (Hille, 1992) and Myxicola (Rudy, 1975). However, the *Polyorchis* SMN sodium current has a $T_{1/2}$ of 52.6 ms from a holding potential of -80 mV, whereas at -40 mV, which is closer to the resting membrane potential, the $T_{1/2}$ was closer to 200 ms (unpublished). These values for $T_{1/2}$ more closely resemble those recorded for non-neuronal cells, for example, mammalian Purkinje cells and heart muscle cells. They have $T_{1/2}$ values of approximately 80-170 ms at room temperatures (Brown *et al.*, 1981a; Colatsky, 1980; Gettes and Reuter, 1974). Other striking physiological similarities of these two systems include: encoding of information in the duration of their action potentials and their slow conduction velocities. Concerning this latter feature, the conduction velocities of action potentials in the SMN network is approximately 1.1 m s^{-1} (Spencer, 1981), whereas the velocity in Purkinje fibers approximates 1.8-3.0 m s^{-1} at 35 - 37 °C (Rosen *et al.*, 1981; Schoenberg *et al.*, 1975). Two parameters, the space constant of the network and the density of sodium channels, have a major influence on conduction velocity in such electrically coupled systems. It is probably significant that the density of sodium channels in these two systems is similarly low (SMN = 0.2 +/- 0.03 mA/cm^2, this study; Purkinje fibres = 0.5-1.0 mA/cm^2) (Fozzard *et al.*, 1986) when compared with systems having high conduction velocities such as a typical unmyelinated giant axon (4 mA/cm^2) (Hille, 1992).

Existence of more than one neuronal sodium current in *Polyorchis*

Using either histological and/or electrophysiological data, at least four distinct neuronal types and associated networks can be identified in the central nervous system (nerve rings) of *Polyorchis* (Grimmelikhuijzen and Spencer, 1984; Spencer, 1981; Spencer and Arkett, 1984). One of these is the B neuron network, which makes excitatory synaptic connections to both SMNs and tentacle smooth muscle. Unlike SMNs, the morphology of B neurons is quite clearly typical of neurons in vertebrates. The long and relatively fine processes of B neurons contrast with the blunt, short processes of SMNs that are more reminiscent of epithelial cells or fibroblasts. In addition, B neurons and SMNs have very different spike shapes and firing patterns. B neurons produce rapid, short, constant duration spikes in a bursting pattern (Spencer and Arkett, 1984) whereas spikes in SMNs are of long and variable duration and the firing pattern is tonic (Spencer, 1981). Several properties of the sodium current that we recorded from B neurons indicate that they possess a channel population that is distinctly different from the channels of SMNs. The slow component of inactivation in B neurons is slower than in SMNs (Fig. 3-8), whereas the rate of recovery from inactivation is considerably faster in B neurons than in SMNs (Fig. 3-9). This rapid recovery from

inactivation permits B neurons to fire spontaneously at frequencies as high as 10 Hz (Spencer and Arkett, 1984).

Thus it is apparent that some diversity existed in sodium channel properties, and presumably structure, early in the evolution of metazoans. Full-length sodium channel sequences have been cloned from the hydrozoan species, *P. penicillatus* (see Chapters 4 and 5) and from species of other cnidarian classes, the scyphozoan *Cyanea capillata* (Anderson *et al.*, 1993) and the anthozoan *Aiptasia pallida* (White *et al.*, 1998). They show an overall match in similarity and identity of approximately 60% and 40% respectively when compared with a representative sample of invertebrate and vertebrate sodium channel genes (see Chapter 4). However the properties of all of these channel proteins are not yet known. Our present data also suggest that there may have been functional assortment of sodium channel subtypes to particular cell lineages in cnidarians such that there are channels specific for true neurons whereas other channel subtypes can be found in excitable, non-neuronal cells. There are likely functional representatives of mammalian isoforms of other major family of voltage-gated channels besides sodium channels. It has been shown recently that genes coding for several of the potassium channel subtypes such as *Shaker* and *Shal* are present in *Polyorchis* (Jegla *et al.*, 1995) and a calcium channel in the scyphozoan *Cyanea capillata* (Jeziorski *et al.*, 1997). We must conclude that there was an explosive diversification of the genes coding for the major voltage-gated channel families at a time before or during the earliest period of metazoan radiation.

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CHAPTER 4 A PUTATIVE VOLTAGE-GATED SODIUM CHANNEL ALPHA SUBUNIT (PPSCN1) FROM THE HYDROZOAN JELLYFISH, *POLYORCHIS PENICILLATUS*: STRUCTURAL COMPARISONS AND EVOLUTIONARY CONSIDERATIONS[5,6,7]

INTRODUCTION

Sodium channels are considered to be the most recently evolved members of the voltage-gated superfamily, and apparently derived from calcium channels with four domains (Strong *et al.*, 1993). Although there are reports of voltage-gated sodium currents recorded from protozoans, the channels identified as being responsible are either poorly selective (calcium vs sodium) or are receptor activated (Hennessey, 1989; Saimi Y. and Ling K.Y., 1995). Development of an ionic specificity for sodium rather than calcium probably resulted from selection for ionic currents that could mediate high frequency signaling and rapid propagation to coordinate the activities of multicellular

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[5] A version of this chapter has been published. Spafford, J.D., Gallin, W.J. and Spencer, A.N. (1998) *Biochemical and Biophysical Research Communications*. 244:3:772-780.

[6] The nucleotide and deduced amino acid sequences reported in this chapter have been deposited in GenBank (Accession #AF047380).

organisms, and thus may have been coincident with the appearance of the Metazoa. Calcium currents are poorly suited to this role since the influx of calcium during spike trains could interfere with intracellular calcium signals and might lead to calcium cytotoxicity (Hille, 1992).

Most evidence suggests that voltage-gated sodium-selective channels from this superfamily appeared in an ancestral metazoan. The cnidarians are one of a group of metazoan phyla that have uncertain relationships at the base of the metazoan phylogenetic tree. The most balanced synthesis of current opinions regarding the phylogeny of the Porifera, Placozoa, Mesozoa, Cnidaria, Ctenophora and Platyhelminthes would place this latter phylum as being basal to all later metazoans with the former five phyla being sister groups with an unknown "planuloid" ancestor of the Platyhelminthes (Willmer, 1990). Thus the cnidarian condition should be partially representative of early evolutionary diversification of voltage-gated channels. To date, cnidarian voltage-gated sodium channel cDNAs have been isolated from the scyphozoan jellyfish *Cyanea capillata* (CYNA1) (Anderson *et al.*, 1993) and the anthozoan *Aiptasia pallida* (AnemNa1) (White *et al.*, 1998).

We have cloned a full-length sodium channel cDNA, from the hydrozoan jellyfish, *Polyorchis penicillatus*, that encodes PpSCN1 (*Polyorchis penicillatus* sodium channel #1, GenBank Accession #AF047380) which based upon structural similarity, is a member of the voltage-gated sodium channel family. Comparisons of PpSCN1 with

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[7]The published GenBank script for PpSCN1 is shown in Appendix A.

aligned sequences of other sodium channels, reveal that many of the residues that are considered to be critical for ionic selectivity, voltage sensing, and binding of TTX and lidocaine in mammals differ in the Cnidaria. PpSCN1 is the only α subunit presently available to account for at least three electrophysiologically identified sodium channel currents in *P. penicillatus*. The apparent absence of structural diversity of α subunits suggests that the functional diversity of sodium channels in *P. penicillatus* may depend on accessory proteins or post-translational modification of α subunits, or that there are other sodium channel families present that are so structurally distinct as not to be isolated by the methods used in this study.

MATERIALS AND METHODS

Collection of Polyorchis penicillatus

Medusae of *P. penicillatus* were collected by SCUBA from Bamfield Inlet, B.C. and held in running seawater for several days until the gut contents were excreted or absorbed.

Design of PCR primers and analysis of DNA and amino-acid sequences

Protein sequences of sodium and calcium channels were retrieved from GENBANK database using BLAST (Altschul *et al.*, 1990). Multiple alignments were made using a modified progressive pair-wise alignment method of Feng and Doolittle (Feng and Doolittle, 1987) in PileUp (GCG, 1995). Gap creation and extension penalties were 3.0 and 0.10 respectively. MacVector 6.0 (Oxford Molecular Group), Gene Construction Kit 2 (Textco, Inc.) and GCG v8.1 (Genetics Computer Group, 1995) were used for alignments, assembly and analysis of DNA and amino-acid sequences.

Degenerate primers were designed based on the consensus regions of published amino acid sequences of sodium and calcium channels (six known at the time). Primers were made if they satisfied the following criteria: a) perfect consensus of known invertebrate clones; b) low or no degeneracy near the 3' end; c) overall degeneracy < 500; d) 17 to 25 bp in length; e) relatively high annealing temperature (calculation based on the G:C content of the primers); f) expected size of PCR product between 100 bp to 2 kB; and g) homologous region in both sodium and calcium channels. In total, 19 sodium channel and 12 calcium channel primers were synthesized using an ABI DNA synthesizer (Perkin-Elmer).

Using PCR to synthesize channel probes

Three sources of DNA were used as a template in the polymerase chain reaction (PCR) to synthesize channel probes: 1) Genomic DNA extracted from jellyfish gonads; 2) First strand cDNA template derived from polyA mRNA extracted from nerve-enriched tissues of *P. penicillatus*; 3) Three oligo-dT and random primed libraries constructed in the lambda Zap II vector (Stratagene, La Jolla). Details of the preparation and construction of cDNA libraries were described previously (Gallin, 1991). The cDNA libraries had a complexity of 2×10^5.

A negative, no template control and a positive control PCR reaction with rat brain II (SCN2A) sodium channel clone were included in each group of experiments.

When no or poor PCR products were recovered, PCR reactions were optimized by adjusting the following variables: a) concentration of $MgCL_2$; b) annealing temperature; c) extension time; d) amount of template; e) number of cycles of the PCR; f) pH of the reaction mix.

The likelihood of the sequenced DNA being putative channel sequence was assessed using the following criteria: 1) The sequencing primer and polylinker sequenced are readable at the front end of the sequence; 2) The sequence contains a possible match with the degenerate primers at both ends of the insert; 3) There is an open reading frame (ORF) with no stop codons after the primer sequence; 4) The deduced amino acid sequence from the ORF sequence has a sequence most similar to known channel clones

when the sequence is compared to known DNA sequences published in GENBANK (search using TBLASTN); 5) The sequence similarity is greatest in the region of the sequence that is most conserved in other known DNA clones; 6) The sequence is more similar to invertebrate clones than vertebrate ones; 7) The sequence is of the approximate size of known channel clones; 8) The DNA x DNA sequence identity with GENBANK gives known channel clones the highest score (search using BLASTN); 9) The DNA sequence is not a perfect match with any known clone; 10) The DNA hybridized to a Northern blot of *P. penicillatus* RNA and yielded expected sized band(s) for a sodium channel.

Using the above criteria, I assessed many clones. As expected from using degenerate primers in the PCR, I had many failures. My first success came more than a year later. It was a single clone that I sequenced and analysed on Christmas eve 1993.

A positive PCR product coding for a sodium channel was obtained using a sense primer (GGNATGCARYTNTTYGG) and an antisense primer (TCDATCCAYTYNCCRCANA) designed from the conserved amino-acid sequence bordering the putative pore region of domain II of published sodium channels. 20 pmol of each primer were used in a PCR reaction (25 µl) with 200 ng *P. penicillatus* genomic DNA as a template, (0.15 units) recombinant Pfu DNA polymerase (Stratagene, La Jolla) and Taq polymerase, 20 mM Tris-HCl (pH 8.3 at 20^{o}C), 25 mM KCl, 100 µg/ml gelatin, 50 µM each dNTP and 2.0 mM $MgCl_2$. Thermal cycles of (93^{o} C, 45 s; 42^{o}C, 1 min.;

68°C, 1 min.) were repeated 5 times then cycles of (93°C, 45 s; 55°C, 1 min; 68°C, 1 min.) were repeated 30 times, followed by 10 min. at 72°C.

The resulting 138-bp DNA fragment from the PCR was too small to hybridize to a Southern or Northern blot of *P. penicillatus* DNA, so a larger fragment was generated using inverse PCR. The 138-bp DNA sequence was used to design two outward-facing primers (GAATTCATTCATGATGATATTTCG and AGCTGCTTCCAATTCG) for inverse PCR reactions. Ten template were prepared by digesting 200 ng *P. penicillatus* genomic DNA with ten different restriction enzyme, circularized with T4 DNA ligase and amplified under the same PCR conditions outlined above. Inverse PCR of a *TaqI*-digested template was the only one to yield a positive PCR product.

The positive PCR fragment (941 bp) was used as a probe to screen cDNA libraries. Duplicate Hybond-N nylon filters (Amersham, Little Chalfont) were lifted from 20 plates with 5×10^4 plaque-forming units per plate. Hybridization was carried out using the method of Church and Gilbert (Church and Gilbert, 1984). Two different positive cDNA clones were isolated by hybridization screening of cDNA libraries (fig. 4-1): PpSCN1.1Ac (3.7 kb) containing the 3' end of the gene and PpSCN1.2Ac (1.7 kb). Positive clones were recovered in Bluescript SK- vector by co-infection with R408 helper bacteriophage (Ausubel *et al.*, 1997).

DNA/mRNA isolation, RT-PCR and 5' RACE

Total RNA and genomic DNA were prepared from bell margins and tentacles of *P. penicillatus* medusae by homogenizing the tissue in TRIZOL Reagent (Life Technologies, Gaithersburg). Poly $(A)^+$mRNA was purified on oligo-(dT) spun columns from an Oligotex mRNA Midi Kit (QIAGEN, Chatsworth). The mRNA was used to synthesize the 5' end of the cDNA by 5' RACE (Chenchik *et al.*, 1996). Approximately 1 μg of poly $(A)^+$ mRNA was reverse-transcribed (RT) with an antisense primer (TCTAGGATAATCTTCACC) based on the sequence near the 5' end of PpSCN1.1Ac, a second strand was synthesized, blunt-ended and a Marathon adaptor (Clontech, Palo Alto) was ligated to the 5' end of the cDNA. PCR was performed with the cDNA template, an antisense primer nested inside the RT-primer (AGCTGCTTCCAATTCGGCT) and either a 5' primer based on genomic sequence (see Chapter 5) (CGCTGTTTTTGTTTGGTCCA) to yield a 1647 bp RT-PCR product (PpSCN1.2Bc) or a 5' primer based on sequence of the Marathon adaptor, yielding a larger 2185 bp 5' RACE product, PpSCN1.1Bc, that encompassed the 5' end of the mRNA (fig. 4-1). PCR (25 μl) was performed with 250 units/ml KlenTaq1 (Ab Peptides, St. Louis) and 1.5 units/ml Pfu DNA polymerase (Stratagene, La Jolla), 20 pmol each primer, 50 mM Tris-HCl (pH 9.1), 16 mM ammonium sulfate, 3.5 mM $MgCl_2$, 150 mg/ml BSA and 50 μM each dNTP in a Perkin-Elmer 9600 Thermal Cycler,

"hot-started" (Ausubel *et al.*, 1997) with DNA polymerase mix added after 94° C, 1 min. followed by thermal cycles of (94° C, 5 sec.; 60°C, 30 sec.; 68°C, 4 min.) repeated 30 times followed by 10 min. at 72°C.

Northern blot analysis, sub-cloning and DNA sequencing

DNA probes were ^{32}P-labeled by incorporation of radioactive nucleotides in the synthesis of DNA in PCR or labeled with random hexamers and the large fragment of DNA polymerase I (Klenow). ^{32}P-labeled probe was hybridized to a Northern blot with 5 μg per lane *P. penicillatus* poly $(A)^+$ mRNA. Northern blots were prepared on Genescreen Plus nylon hybridization transfer membrane (Dupont NEN, Boston) with hybridization conditions and protocol as described above for library screening (Church and Gilbert, 1984).

PCR and cDNA fragments were digested with restriction enzymes into smaller sized fragments (300-500 bp), subcloned into Bluescript KS+ vector and sequenced in both directions using a Perkin-Elmer ABI 373A sequencer and an ABI Prism Dye-Terminator Cycle Sequencing Kit. PCR products and gel-extracted DNA were purified using silica-based DNA technology (Hengen, 1995) with an additional purification for sequencing through QIAGEN-tip 20 columns (QIAGEN, Chatsworth).

RESULTS AND DISCUSSION

Cloning and sequencing of the putative sodium channel gene from *P. penicillatus*

Fragments of the sodium channel gene PPSCN1 were isolated from *P. penicillatus* genomic DNA amplifications in PCR using degenerate and inverse PCR primers. A 941 bp PCR fragment was used to isolate two cDNA fragments (PpSCN1.1Ac and PpSCN1.2Ac in fig. 4-1), the former being an oligo-dT primed cDNA with the 3' end of the gene and an AATAA consensus sequence upstream from a 32 bp terminal poly (A) tail. The cDNA and genomic sequences (see Chapter 5) were used to design primers for synthesizing the 5' end in RT-PCR (PpSCN1.2Bc in fig. 4-1) and 5' RACE (PpSCN1.1Bc in fig. 4-1). Together, PpSCN1.1Ac and PpSCN1.1Bc form a full-length cDNA with 241 bp and 448 bp, 5' and 3' untranslated regions respectively and a 5085 bp open reading frame. Position 242 is considered the start site because it is the first methionine in open reading frame containing an amino acid sequence that aligns well with other known sodium channels. A Kozak consensus sequence is not present immediately 5' to the start site.

The sequence of genomic DNA that spans the sodium channel cDNA (Accession PPSCN1GEN, #AF047379) (see Chapter 5) confirmed the cDNA sequence. Overlapping partial fragments PpSCN1.2Ac and the PpSCN1.2Bc translate into identical amino acid sequences, but differ at 15 positions, all 15 changes being synonymous substitutions in the third position of the codon (degenerate code in fig. 4-2). These differences are likely due to polymorphisms since tissues were pooled from many jellyfish to construct the cDNA. Only one of the genomic clones, PpSCN1.1g was completely sequenced.

Since only one complete cDNA construct was sequenced (PpSCN1.1Ac and PpSCN1.1Bc) and only partial overlapping fragments of other clones (PpSCN1.2Ac and PpSCN1.2Bc), many more than the 15 polymorphisms reported here likely appear in PpSCN1. Since the genomic clone PpSCN1.2g was sequenced in a strategic manner to confirm that the differences between the two cDNAs were due to polymorphisms rather than errors introduced by the molecular cloning process (eg. *Taq* polymerase in the PCR, misreading sequence data), likely many other differences could be catalogued between the genomic clones, including the non-coding regions of the gene.

Genomic clones PpSCN1.1g and PpSCN1.2g were two out of sixteen positive clones that were isolated from genomic libraries in lambda phage. Many of the clones were likely replicates of one another because the clones came from amplified genomic libraries. Six out of the sixteen positive clones were purified and mapped by restriction enzyme analysis.

Position 1246 in the full-length cDNA of PpSCN1 served to distinguish between different populations of sodium channel clones isolated from *P. penicillatus*. Position 1246 in PpSCN1.1Ac is a guanosine residue which contributes to the six residue consensus of a HpaI restriction site. This guanosine residue in PpSCN1.1Ac is an adenosine in PpSCN1.1Bc. Consequently, PpSCN1.1Bc is not recognized by the HpaI enzyme at this site. Four of the six genomic clones that were mapped appeared to contain the HpaI site and two did not. One of these clones which contained the HpaI site was completely sequenced (PpSCN1.1g). PpSCN1.2g was sequenced for comparison because it did not contain a HpaI site at position 1246. A more thorough study involving sequencing multiple full-length cDNAs and genomic clones will confirm whether the 1) genetic variation found between the two cDNAs corresponds between the two genomic clones described in this thesis; 2) whether there exists more than fifteen polymorphic sites in the coding region of the two genes.

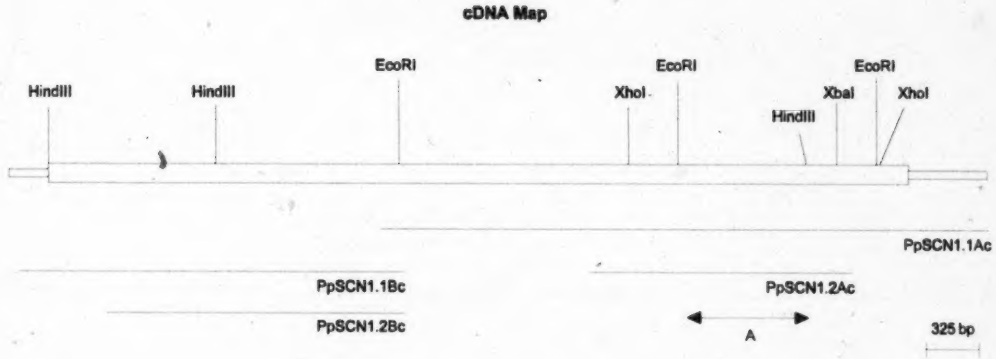

Figure 4-1. Restriction map of the cDNA transcript for PpSCN1. A large open box represents the coding region and the 5' and 3' untranslated regions by smaller boxes. Below the map, the extents of the individual cloned fragments are indicated by lines and the cDNA probe used in the Northern blot by fragment A (with arrowheads). PpSCN1.1Ac and PpSCN1.2Ac were isolated from cDNA libraries by hybridization screening. PpSCN1.1Bc and PpSCN1.2Bc are 5'RACE and RT-PCR products respectively.

```
TTATGTAAGTTATTTTTAAGTAGATTATTTTCTACCAATAGACATGAGAGAAACATTTTT    60
TAAATAATGAGTTGCTTTCGTCATAGGATGCATTTTACCTCTTAACTTATTTCTGTACGA   120
AAGTATATATTATTATTGTAGTCGCTGACACATAATCAGAAGTCAACAAAGACTCCAGCTTTT 180
TGTCAAATTTTGGTTAGATTATAACGATTTCATAGAATTAGGATGTCAAAGAACATTCCA   240
AATGAAGCTTTCCTTCTCTACTGAATGGCTAAAAATTAAAAAAAATATGTTACAAAATCT   300
  M  K  L  S  F  S  T  E  W  L  K  I  K  K  N  M  L  Q  N  L    20
           *
TTTTATGACGCACTCATTAAATAATGTAGGACTGACTGTTTTTTATTTAGGACAATCGAC   360
  F  M  T  H  S  L  N  N  V  G  L  T  V  F  Y  L  G  Q  S  T    40

AGTCATATTAAATGCTTTCTATTTTGGAATAGTTTTTAATAAACAGAAAAAAAAAAGTCC   420
  V  I  L  N  A  F  Y  F  G  I  V  F  N  K  Q  K  K  K  S  P    60
                                                    *
ACACTTTTTTCACTTCTCACACGGCCGTCCCACAAAATTTATTTTTGGATTTTTCACATT   480
  H  F  F  H  F  S  H  G  R  P  T  K  F  I  F  G  F  F  T  F    80
                          *
TATGGTGGTTGCTAATAAATTGGGAAAACAAGTCGTTTTTCGTTTTTGTAAAGAAAAATC   540
  M  V  V  A  N  K  L  G  K  Q  V  V  F  R  F  C  K  E  K  S   100

GCTGTTTTTGTTTGGTCCAGCAAGTTTYATTCGTAGATTCAAYATATATATTTTTACTCA   600
  L  F  L  F  G  P  A  S  F  I  R  R  F  N  I  Y  I  F  T  H   120

TCAATATTTTGAAATAGGGATTTTATTGACAATTGTAACCAACTGTGTGTTTATGGCGCT   660
  Q  Y  F  E  I  G  I  L  L  T  I  V  T  N  C  V  F  M  A  L   140
                    IS1
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- Figure 4-2 continues on next page -

Figure 4-2. Nucleotide sequences of PpSCN1 cDNA (upper) and its deduced amino acid sequence (lower). The predicted protein shares the topology of typical sodium channels, consisting of four homologous domains (I, II, III, IV) connected by inter-domain, cytoplasmic linkers. Each domain has six hydrophobic, transmembrane segments S1 through S6 (underlined), with a voltage-sensing segment in S4 where positive charged (R/K) residues repeat every third amino-acid along the segment (thick-underlined residues) and a membrane-penetrating, pore-forming loop (P loop) between S5 and S6 (short-segments 1 and 2, wavy underlining). There are 25 potential sites for phosphorylation by protein kinase C (* below residues) and 5 sites for protein kinase A (residues with inverted color). PpSCN1 has 8 possible N-linked glycosylation sites predicted to be extracellular (^ below residues, numbered 1 to 8). Six out of the eight potential glycosylation sites in PpSCN1 (numbered 1,2,3,5,6 and 8) are conserved in homologous positions in almost all sodium channels. Alignment of the extracellular loops reveals that sites 2, 5 and 3, 6 are in homologous positions in extracellular loop of the pore in domains I and III. Their preservation in the same position in domains I and III suggest that these N-glycosylation sites were likely present in the extracellular loop of the first of two domains, in the two-domain ancestor.

```
AAGTGATCCRCCAGCAGAAGCAGAATTTATTTTTGCTGCCATATATACTTTTGAAATGGT    720
  S  D  P  P  A  E  A  E  F  I  F  A  A  I  Y  T  F  E  M  V    160
                                                   IS2
TATAAAGATACTAGCTAAAGGTTTTATACTACATAAATATTCTTATTTGAGAAATGCATG    780
  I  K  I  L  A  K  G  F  I  L  H  K  Y  S  Y  L  R  N  A  W    180
                                                   *
GAATTGGCTTGATTTCCTGGTTGTTATACTAGGGTATATTACCATGTCTCCACACATCAA    840
  N  W  L  D  F  L  V  V  I  L  G  Y  I  T  M  S  P  H  I  N    200
                      IS3
TAATTTGAGTGGAATAAGAACGTTTCGTGTACTGCGAGCTTTRAAAACCATATCCACTGT    900
  N  L  S  G  I  R  T  F  R  V  L  R  A  L  K  T  I  S  T  V    220
  ^1                                  IS4
CAAAGGTTTAAAAGCAATGGTTAATACATTAATGAAGTCCATGAAAATGATGACTGATGT    960
  K  G  L  K  A  M  V  N  T  L  M  K  S  M  K  M  M  T  D  V    240
                                      *
TCTAATATTAACGCTGTTTTTCATATCAATATTTGCCTTGATTGGTTTACAGCTATTCCC   1020
  L  I  L  T  L  F  F  I  S  I  F  A  L  I  G  L  Q  L  F  P    260
                      IS5
TGGTAAATTAAGACAACGTTGTGTTCTGAAAAATGGTAACAAAACYTTCGAAAAATCATG   1080
  G  K  L  R  Q  R  C  V  L  K  N  G  N  K  T  F  E  K  S  W    280
                                      ^2
GTATACTAATAAAGATAAYTTTTTGTATTTCGAAAARGAAGAAGTTATTTGTGGAAATGA   1140
  Y  T  N  K  D  N  F  L  Y  F  E  K  E  E  V  I  C  G  N  E    300

AACAACGTCATGGAAATGTGACGCTGGTTATATTTGCTTAGCTGATGCAGGTAATAACCC   1200
  T  T  S  W  K  C  D  A  G  Y  I  C  L  A  D  A  G  N  N  P    320
  ^3
GATGCATGGTTATGTCAGTTATGATAATTTCCTAAGTGCCATGTTRACAAGCTTGCAAGT   1260
  M  H  G  Y  V  S  Y  D  N  F  L  S  A  M  L  T  S  L  Q  V    340

TTGCACTTTAGATTATTGGGAGAGTGTTTTTAACAGTGTCCTTTCAGCTATGGGAGAGCC   1320
  C  T  L  D  Y  W  E  S  V  F  N  S  V  L  S  A  M  G  E  P    360

ATATATGATTTATTTTTTACTGGCAGTATTCCTGGGACCATTTTACCTTTTAAATCTGGT   1380
  Y  M  I  Y  F  L  L  A  V  F  L  G  P  F  Y  L  L  N  L  V    380
                      IS6
ACTTGCAGTTGTTAGTGCTTCGTATGAAGCAGAAGTTAACGGGAATCCAGATGAAGAAGT   1440
  L  A  V  V  S  A  S  Y  E  A  E  V  N  G  N  P  D  E  E  V    400

GGAACGGGAGAACATGGCTAAAATACGGAGAAGTGCTTCCGCATATTCTTTYGACGGTGA   1500
  E  R  E  N  M  A  K  I  R  R  S  A  S  A  Y  S  F  D  G  E    420
                                   *
ATATTGTGTTGAATACTTAACTGGTCCATCTCCAGTAGAAGAAATTGACGGTGAAAAAAG   1560
  Y  C  V  E  Y  L  T  G  P  S  P  V  E  E  I  D  G  E  K  R    440

ATACACTATACCTATTGAAGGTTCAAAAAAGAAAAAAAACAAAGATGTTGATGATGATTT   1620
  Y  T  I  P  I  E  G  S  K  K  K  K  N  K  D  V  D  D  D  L    460
     *              *
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- Figure 4-2 continues on next page -

```
ACAGCTACCACCAAAATTAGGAGACAATCCTACCATGAAGATTCGAGTGCAATACTTTTT  1680
  Q  L  P  P  K  L  G  D  N  P  T  M  K  I  R  V  Q  Y  F  F   480
                                   *

TTTTGTATTTGTATCAAGTTCTGCATTTGAAGGCTTCATTACATTCTGTATAATGCTAAA  1740
  F  V  F  V  S  S  S  A  F  E  G  F  I  T  F  C  I  M  L  N   500
                                  IIS1

CACATGTTTAATGGCATCGGAACATTACAAAATGCCAAAAAAAATTGGAAGATGCACTAAA  1800
  T  C  L  M  A  S  E  H  Y  K  M  P  K  K  L  E  D  A  L  N   520

TGTTTTTAATTATATATTTACAGCGACCTTTTGTATTGAAATGGGATTTAAAATACTAGC  1860
  V  F  N  Y  I  F  T  A  T  F  C  I  E  M  G  F  K  I  L  A   540
                                  IIS2

CTTTACTCCAAAAGGTTATATACGAAATAGATGGAATGTCTTTGATGGTTTGCTGGTGAT  1920
  F  T  P  K  G  Y  I  R  N  R  W  N  V  F  D  G  L  L  V  I   560
     *                                                  IIS3

TGTGAGCATTATTGATATAGTGTTATCCAACGCTGATGTTGTAAAGGGAAATCAACTAGC  1980
  V  S  I  I  D  I  V  L  S  N  A  D  V  V  K  G  N  Q  L  A   580

TGTGTTAAAAGTGTTTCGCTTAATGCGGGTATTAAAATTAGCACAGTCTTGGAAAACAAT  2040
  V  L  K  V  F  R  L  M  R  V  L  K  L  A  Q  S  W  K  T  M   600
                                  IIS4

GGGTCAATTACTGAGCACAATAGCAAGTAGCATGGGTGCTCTTGGGAACGTCACAGTTAT  2100
  G  Q  L  L  S  T  I  A  S  S  M  G  A  L  G  N  V  T  V  I   620

TCTAGGTCTAATCATTTATATTTTTTCTGTTGTCGGCATGCAGCTGTTCGGKAAATATTA  2160
  L  G  L  I  I  Y  I  F  S  V  V  G  M  Q  L  F  G  K  Y  Y   640
                     IIS5

CAATAAAGCCGAATTGGAAGCAGCTGGTGAAGATTATCCTAGATACAATTTTGAAAATTT  2220
  N  K  A  E  L  E  A  A  G  E  D  Y  P  R  Y  N  F  E  N  F   660

TGGGAATTCRTTCATGATGATATTTCGTATACTATGTGGCAAATGGATAGAACCACAATG  2280
  G  N  S  F  M  M  I  F  R  I  L  C  G  K  W  I  E  P  Q  W   680

GGATTTACTAAATAGAACAAATCCGGCGTCTATACTGTATATTTTTTTTGTTTTTGTTAT  2340
  D  L  L  N  R  T  N  P  A  S  I  L  Y  I  F  F  V  F  V  I   700
           ^4

TGGTAGATGGGTGGTACTCAATTTGTTTTTGGCTTTATTACTAAGTTCATTTGGTGGCGA  2400
  G  R  W  V  V  L  N  L  F  L  A  L  L  L  S  S  F  G  G  D   720
        IIS6

TGCATTAAATGGCGGCGGAGATGATGATTCGGAAAAACCCAAAAAAAGCAGATTAAAACG  2460
  A  L  N  G  G  G  D  D  D  S  E  K  P  K  K  S  R  L  K  R   740
                              *                 *

TTTAATAGACTGGACTCGTAAAAAGAGAAACAAAAAATCAAAAACATGTATGGTCTCTGG  2520
  L  I  D  W  T  R  K  K  R  N  K  K  S  K  T  C  M  V  S  G   760
           *                             *

ACCACCCGAAGATCTGGAACTTTACACAGGTGAAATGATAGTGCTAGATAATAATGGAAA  2580
  P  P  E  D  L  E  L  Y  T  G  E  M  I  V  L  D  N  N  G  N   780
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- Figure 4-2 continues on next page -

```
TTCTCATAAGAATGGTGACATTGAACAAAATGACAATTATTTAAAGCCAGAATTTCCTCG   2640
  S  H  K  N  G  D  I  E  Q  N  D  N  Y  L  K  P  E  F  P  R    800

TCATGACTCGATACTATCGTCTTTACCGAACGGTATATCACTAACTAATGGAGATTTATC   2700
  H  D  S  I  L  S  S  L  P  N  G  I  S  L  T  N  G  D  L  S    820
                                                          *

TAAAAGACAGATTTCGGGTTCAGTAGATATAGATATTGCGTTGTCTTTAAACAGCGATAA   2760
  K  R  Q  I  S  G  S  V  D  I  D  I  A  L  S  L  N  S  D  N    840

TGCAGTTAGTAACAAAACAATGAGTATTCATCCACAATCGTCAGCATCTGTAAAAATTGA   2820
  A  V  S  N  K  T  M  S  I  H  P  Q  S  S  A  S  V  K  I  E    860
        *

AATTCAACGTGAAAAGACTTATGTTGATGACTGCCTATGTACACTTTGTTACCAATGTTC   2880
  I  Q  R  E  K  T  Y  V  D  D  C  L  C  T  L  C  Y  Q  C  S    880

TTGTTGTTACACCAGTTACTTAAATGCGCCGTTTAGACGGTCGTGGCATAATGCAAGATT   2940
  C  C  Y  T  S  Y  L  N  A  P  F  R  R  S  W  H  N  A  R  F    900
                                          *

TTACACCAAACAGTTAATTGAGCATAAATATTTTGAAGGCGTTATTTTGTTTCTTATAGC   3000
  Y  T  K  Q  L  I  E  H  K  Y  F  E  G  V  I  L  F  L  I  A    920
                                                   IIIS1

TTTTAGTAGCTTGACCTTAGTATTTGAAGACATTGACTTACCTAAGCGTCCTACTTTAGA   3060
  F  S  S  L  T  L  V  F  E  D  I  D  L  P  K  R  P  T  L  E    940

AAAGTTTTTACAATATTGTAATTACTTCTTCGCTGTCATATTTACAGTGGAATTACTTAT   3120
  K  F  L  Q  Y  C  N  Y  F  F  A  V  I  F  T  V  E  L  L  I    960
                                             IIIS2

CAAGCTATTTGCACTTGGTTTCATGAAATACTTTACAAACTTTTGGAATTTACTTGATGT   3180
  K  L  F  A  L  G  F  M  K  Y  F  T  N  F  W  N  L  L  D  V    980

CTTCATTGTGTGTATTTCATTGTCTTCGCTCTTCGGTAAAGGTGCAAATTTAAAAGCGTT   3240
  F  I  V  C  I  S  L  S  S  L  F  G  K  G  A  N  L  K  A  L   1000
        IIIS3

ACGTTCCTTAAGGGGTTTGCGACCTCTGAGGGCGATATCAAGATTCGAAGGAATGAAAGT   3300
  R  S  L  R  G  L  R  P  L  R  A  I  S  R  F  E  G  M  K  V   1020
                    IIIS4

GGTCGTCAACGCGTTGTTATACTCAATACCCTCAATTGCAAATGTATTGCTAGTGTGCGT   3360
  V  V  N  A  L  L  Y  S  I  P  S  I  A  N  V  L  L  V  C  V   1040

AGTATTCTGGTTAATATTTAGCATTATGGGATATAATCTGTTTGGTGGACAGTTCTTTTA   3420
  V  F  W  L  I  F  S  I  M  G  Y  N  L  F  G  G  Q  F  F  Y   1060
        IIIS5

TTGTGTGGATGGGAATTATACGAAACTACCTATTAGTGTGATTGACAACATGGCAATGTG   3480
  C  V  D  G  N  Y  T  K  L  P  I  S  V  I  D  N  M  A  M  C   1080
                ^5

TTTGAATGAAACGAACAAAGCTAAAAATTATTCATGGGTGAATAAAAATATCAACTTTGA   3540
  L  N  E  T  N  K  A  K  N  Y  S  W  V  N  K  N  I  N  F  D   1100
     ^6                    ^7
```

- Figure 4-2 continues on next page -

```
TAATTCGATTAACGGATTTTTAGCTTTGTTTCAAACGGCGACATTGGAAGGATGGTTTGA   3600
  N  S  I  N  G  F  L  A  L  F  Q  T  A  T  L  E  G  W  F  E   1120

AGCTATGGCTGATGCTTATGACTCGAGAGGTGTTAACATGCAACCTGGTGTTATGGTAAA   3660
  A  M  A  D  A  Y  D  S  R  G  V  N  M  Q  P  G  V  M  V  N   1140

CTTTTGGAACCAGATATATTTTGTGGTGTTTATTATACTTGGTGCATTCTTTATTTTAAA   3720
  F  W  N  Q  I  Y  F  V  V  F  I  I  L  G  A  F  F  I  L  N   1160
                          IIIS6

TTTATTCATCGGTGTCATCATTGATAATTTTAATCGGTTAAAACAACAGTACGAAGACGG   3780
  L  F  I  G  V  I  I  D  N  F  N  R  L  K  Q  Q  Y  E  D  G   1180

CGTGGGAATATTTCTGACACCTGGCCAACGGAATTGGGTTAACACTTTGAAAGCTGCCTC   3840
  V  G  I  F  L  T  P  G  Q  R  N  W  V  N  T  L  K  A  A  S   1200

GTTAAAAAAACCCTCAAGAAGACTAACAAGGCCTACGAGCAAATGGAGAGCAGCATTATT   3900
  L  K  K  P  S  R  R  L  T  R  P  T  S  K  W  R  A  A  L  F   1220
                   *                 *

TGATTTTATACATACTAAATATTTCGAATTCTTTATCATGTCAGTTATTTTATTAAACAT   3960
  D  F  I  H  T  K  Y  F  E  F  F  I  M  S  V  I  L  L  N  M   1240
                          IVS1

GCTCACTATGATGATCCAACATCATGGACAAAGTCAACAAGTATCACTTGCCTTAGAATA   4020
  L  T  M  M  I  Q  H  H  G  Q  S  Q  Q  V  S  L  A  L  E  Y   1260

TTTGAACTTTTTATTTACTGGCATATTCACGCTTGAAGCCATTATTAGATTAACTGCTAT   4080
  L  N  F  L  F  T  G  I  F  T  L  E  A  I  I  R  L  T  A  M   1280
                   IVS2                          *

GAGATTGGAGTATTTCAAATATGGAATGAATGTATTTGATTTTGTTATTGTTGTCTTCTC   4140
  R  L  E  Y  F  K  Y  G  M  N  V  F  D  F  V  I  V  V  F  S   1300
                                                IVS3

GATTGCAGTGATAATAATGATCGAGTATGACGAAGAATTTTTCGTTTCACCTGGTTTATT   4200
  I  A  V  I  I  M  I  E  Y  D  E  E  F  F  V  S  P  G  L  F   1320

TCGTGTTGTGCGAGTATTCAGATTAGGACGTTTATTAAGGTTTTTTGAAGGAGCTAAAGG   4260
  R  V  V  R  V  F  R  L  G  R  L  L  R  F  F  E  G  A  K  G   1340
                          IVS4

TATCAGAAAACTCTTATTTACAATTGTAAAATCTGCACCAGCATTAAGCAATATTGGTAC   4320
  I  R  K  L  L  F  T  I  V  K  S  A  P  A  L  S  N  I  G  T   1360

ATTATTGTTTTTGATAACATTTATATACGCTATAATGGCTATGAATCTATTTGGAACATT   4380
  L  L  F  L  I  T  F  I  Y  A  I  M  A  M  N  L  F  G  T  L   1380
                   IVS5

GGCACATCAGGGTGCTATTAATAAAGTGACTAACTTCGAAACATTTGGCCGAAGTATGTG   4440
  A  H  Q  G  A  I  N  K  V  T  N  F  E  T  F  G  R  S  M  C   1400

TTTACTGTTTCGTATATCAACAGCGGCCGGCTGGAATGGTGTACTAGATGCTGCTATGGT   4500
  L  L  F  R  I  S  T  A  A  G  W  N  G  V  L  D  A  A  M  V   1420
```

- Figure 4-2 continues on next page -

```
AGGACCTCCTTTATGCAATGTCAATGCTACCCCAAGTAGTAGTATAAGTGAAGGAAACTG   4560
  G  P  P  L  C  N  V  N  A  T  P  S  S  S  I  S  E  G  N  C   1440
                     ^8
CGGTAATAAATTCGTTGCAGTAATATTTTTTGTATCGTATATAATTTTAATTGTCCTGAT   4620
  G  N  K  F  V  A  V  I  F  F  V  S  Y  I  I  L  I  V  L  I   1460
                                                  IVS6
TATTATAAACATGTATATAGCTGTTATACTGGAAAACTTTAACCAAGCTCAATCACAAGA   4680
  I  I  N  M  Y  I  A  V  I  L  E  N  F  N  Q  A  Q  S  Q  D   1480

TGAAGCTGGCATTACYGAAGACGATTTAGAAGCTTATTAYACMGTTTGGGAAGACTATGA   4740
  E  A  G  I  T  E  D  D  L  E  A  Y  Y  T  V  W  E  D  Y  D   1500

TCCYAAAGCCACCCAATTTATAAAATATTCCCAATTACCCGATTTTATTGACGCTTTAGA   4800
  P  K  A  T  Q  F  I  K  Y  S  Q  L  P  D  F  I  D  A  L  D   1520
           *                 *
CGGTCCATTAAGGGTACCGAAGCCTAATTATTGGTTTTTAGAACAAAGTGATATAGCGAT   4860
  G  P  L  R  V  P  K  P  N  Y  W  F  L  E  Q  S  D  I  A  I   1540

AAAAGATCGACATAGGTGTCATTGTCTAGACGTAATGACCGCTTTGATTAAAAGAGCTTT   4920
  K  D  R  H  R  C  H  C  L  D  V  M  T  A  L  I  K  R  A  L   1560

GGGCGAAGATAACTGCAATGAAAACGAAGATATAAGAAGTGTCATGAAAAAAGTAGAAGA   4980
  G  E  D  N  C  N  E  N  E  D  I  R  S  V  M  K  K  V  E  D   1580

TCGATATAAACAGATTTTTCCACAACGAGCGAAAGAAATAACTAAAGAGACAACACGTGA   5040
  R  Y  K  Q  I  F  P  Q  R  A  K  E  I  T  K  E  T  T  R  D   1600
                                                  *
CCGTTTAAAAATCGAAAACTCGGCTGCCCGTCGTATTCAACGTATATTTCGGAGGCACAT   5100
  R  L  K  I  E  N  S  A  A  R  R  I  Q  R  I  F  R  R  H  I   1620

CCTGATGGATGAAATTTACCAAATGACGAATTCAAGAACAATGAGTTTAAGAGCTCGAGA   5160
  L  M  D  E  I  Y  Q  M  T  N  S  R  T  M  S  L  R  A  R  E   1640
                                             *
AAAAAATTTAAACAAAATTGAACAGCTAGTTACTGTTATGTGGAAAACTCAAAAAAATAA   5220
  K  N  L  N  K  I  E  Q  L  V  T  V  M  W  K  T  Q  K  N  N   1660
                                          *
TTTAATAGAGGAAGGGGATGACGAAAGTGAAGACAACGTCGATGAAAAACAAGACGTTGA   5280
  L  I  E  E  G  D  D  E  S  E  D  N  V  D  E  K  Q  D  V  E   1680

AGAAAGAGAAGAAGAGGAAATGGATGAAGAAAAGAAAGAAGCTAACTAAGTGTTGTTCAT   5340
  E  R  E  E  E  E  M  D  E  E  K  K  E  A  N  -               1695

TTTTGTTGGAAGTTGCCAAATCCAATAGGGCGGATGAAAATTTAAACAGAATTGATTTAA   5400
AGAAATGTCCGTAAGTAAAAACAAAAGCTCACGTAAAAACATTTAACATAAACCAGTCAT   5460
TTAAAAAACGAGAACAAGTAGATAAATGTGTTAGTAATTCGATTAAAAAGAGTTTATGTA   5520
AATGACAGGGATGTGACTTCATGTAAGAAAGATTCATGGAATAAATATCACCATATATAT   5580
ATGTACATATATATATATATATATATATATATAAACCTGTACGCAACTTCACAGATGTAC   5640
TTGTAAAGTACAAAACTAGCTACTGAATGTATTCCTGAAATACAACAGTCTAACAACAGA   5700
ATTTATGTACACATGTATTCTATGTCATTTTGTTTTCAAATGTTTTTATAATATCTACAC   5760
CGTTGCTGTTTTTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                 5806
```

Topology and structure-function considerations for the sodium channel protein (PpSCN1)

The deduced protein sequence of PpSCN1 has one of the smallest coding regions of known sodium channels with four domains, having a coding region of 1695 amino acids and a calculated molecular mass of 193 kDa. Omitting the termini and large loops and considering the major membrane-spanning domains and short cytoplasmic linker between domains III and IV, there is a high degree of similarity (~70%) between PpSCN1 and representative sodium channels (Table 4-1). These conserved regions are implicated in the fundamental properties of sodium channels: selectivity, voltage-sensing, activation and inactivation (Catterall, 1996). The high degree of similarity among such evolutionarily distant species, cnidarians, insects and mammals, suggests that there must have been strong functional constraints on these regions. In comparison, the amino- and carboxyl-termini and inter-domain linkers between domains I and II and between domains II and III, and the extracellular loop in domain I, vary considerably more in sequence and size (Table 4-1). Variable regions in sodium channels that are exposed to the cytoplasm are reported to have structural motifs that may serve as phosphorylation sites for protein kinases, associate with accessory channel subunits and bind cytoplasmic proteins (Adelman, 1995). It is likely that, in the absence of strong functional constraints, these variable regions have evolved to meet the unique requirements of cytoplasmic regulatory environments in various tissues.

Table 4-1

Comparisons Between Predicted Protein Regions in PpSCN1 with Similar Regions in Representative NaChannel Amino Acid Sequences

			Conserved Regions*			Unconserved Regions**		
Channel Description	GenBank Identifier	Coding Region (amino acids)	%Similarity	%Identity	Size (amino acids) (Ratio / PpSCN1)	%Similarity	%Identity	Size (amino acids) (Ratio / PpSCN1)
hydrozoan	PpSCN1	1895			967 (1.00)			928 (1.00)
scyphozoan	CYNA1	1739	85.51	75.66	963 (1.00)	66.77	48.26	776 (1.07)
anthozoan	AnemNa1	1810	76.59	56.31	964 (1.00)	50.53	33.24	846 (1.16)
Drosophila fly	*Para* locus	2157	69.82	45.89	962 (0.99)	49.02	26.91	1195 (1.64)
human skeletal	SCN4A	2016	70.35	46.48	971 (1.01)	48.70	24.73	1045 (1.43)
human heart	SCN5A	1836	70.59	48.00	973 (1.00)	46.58	24.95	863 (1.19)

*averages were calculated using n=5 conserved regions. These conserved regions are:
membrane-spanning Domains 1 to 4 (minus the extracellular loop in D1) plus the cytoplasmic linker between D3-D4

** averages were calculated using n=4 conserved regions. These unconserved regions are:
amino and carboxyl termini, extracellular loop in DI and cytoplasmic linkers between DI and D2 and between D2 and D3

PpSCN1 shows an overall match in similarity and identity of 62.1% ± 0.3 % and 39.5% ± 0.4 % respectively when compared with a representative sample of 12 invertebrate and vertebrate sodium channel genes[8]. Together PpSCN1 and the other cnidarian sodium channel clones from a scyphozoan jellyfish (CYNA1) (Anderson *et al.*, 1993) and an anthozoan (AnemNa1) (White *et al.*, 1998) form a three-gene subgroup within the sodium channel family, with an overall match in amino-acid similarity / identity of 77.4% / 62.8%, and 65.8% / 47.0%, respectively. This sub-grouping can be expected considering that the Cnidaria are thought to have branched quite early from both the protostome and vertebrate lineages, some 800-1000 million years ago (Morris, 1993).

Although we cannot know the exact relationship between cnidarians and the ancestor in which calcium-selective channels first began to show a specificity for sodium ions, extant sodium channels in cnidarians may show traces of this evolutionary modification. Both calcium-based (Anderson, 1989; Dunlap *et al.*, 1987; Holman and Anderson, 1991; Mackie and Meech, 1985; Przysiezniak and Spencer, 1992) and sodium-based (Anderson, 1987; Anderson and McKay, 1987; Grigoriev *et al.*, 1996a; Holman and Anderson, 1991; Mackie and Meech, 1985) action potentials have been recorded in a variety of cnidarians. Thus it would not be surprising if a four-domain channel were identified in *Polyorchis* that was intermediate between sodium channels and calcium

[8] GenBank Accession #s: X14395, D14525, U93074, D17311, Y09108, M77235, U26707, D37977, M81758, X01119, M32078, L19979

channels. However, PpSCN1 has only limited sequence similarity to calcium channels[9] with similarity / identity of 55.0% ± 0.3% and 28.8% ± 0.2% (n=14).

Four negatively charged, glutamate (E) residues, one in the P-loop of each domain, contribute to form a selectivity filter for calcium (boxed amino acids, fig. 4-3A). In sodium channels two out of four of these negatively charged amino acids are replaced. From flatworms to humans, the modified glutamate residues are in domains III and IV, and are replaced by a positively charged lysine residue in domain III and a neutral amino-amino acid, either alanine or glycine, in domain IV (fig. 4-3A). Cnidarian sodium channels PPSCN1 and CYNA1 (Anderson *et al.*, 1993), on the other hand, have a positively charged lysine in domain II and an alanine in domain IV, but the glutamate residue in domain I and III remain unchanged (fig. 4-3A). This places the negatively-charged amino acids opposite one another on either side of the pore rather than being adjacent as in other sodium channels. The only known sodium channels that break this two-negative charges rule for the selectivity filter are channels from squid optic lobe (Heinemann *et al.*, 1992) and sea anemone (White *et al.*, 1998) (fig. 4-3A) that have negative charges in all but domain IV of the sodium channel.

[9] GenBank Accession #s: X99897, D14157, L12531, L15453, D86600, U55776, U25119, U93075, L29529, D38101, L33798, M62554, U00690, AF023602

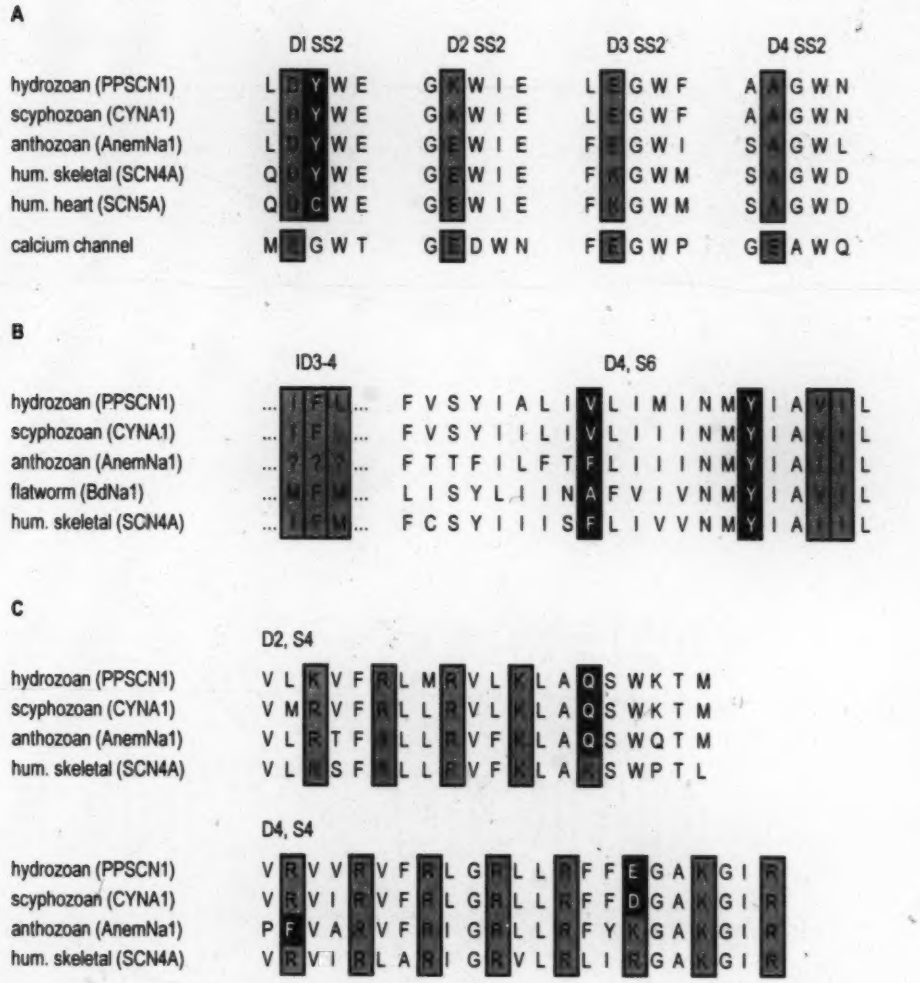

Figure 4-3. Alignments of functionally significant regions of PpSCN1 and homologous amino-acid residues from representative genes of: A) short segment 2 (SS2) of domains I to IV; B) selected residues of the cytoplasmic linker between domains III and IV (IDIII-IV) and the complete segment 6 of domain IV; C) segment 4 of domains II and IV. sodium channel gene sequences: scyphozoan jellyfish (CYNA1), anthozoan sea anemone (AnemNa1), turbellarian flatworm (BDNA1), human skeletal muscle (SCN4A) and human heart (SCN5A) have the following Accession #s in GenBank: L15445, AF041851, U93074, M81758 and M77235, respectively. A human calcium channel gene (CACNL1A1), Accession # L29529, is shown in fig. 4-3A.

Close to the residues responsible for ion-selectivity in the P-loop of domain I is a hydrophobic residue that is considered to be responsible for tetrodotoxin (TTX) sensitivity. Tyrosine (Y) or phenylalanine (F) at this position confers extreme TTX sensitivity (nanomolar range) to mammalian brain or skeletal muscle sodium channels (inverted sequence, fig. 4-3A). Mutagenesis of this site in the skeletal muscle sodium channel to a cysteine (C), as is found in cardiac TTX-resistant sodium channels, produces a 730-fold increase in TTX resistance (Backx *et al.*, 1992), whereas, the reverse (C to Y) mutation in the cardiac channel increases TTX sensitivity (Satin *et al.*, 1992). Cnidarian channels, PpSCN1, AnemNa1 (White *et al.*, 1998) and CYNA1 (Anderson *et al.*, 1993), have tyrosine residues at this position creating a site that should be sensitive to TTX. However, all sodium currents recorded in coelenterates, including species from all three cnidarian classes and a ctenophore, are insensitive to TTX, even at concentrations of 100 µmol (see Chapter 2) (Anderson, 1987; Anderson and McKay, 1987; Dubas *et al.*, 1988). Current models incorporate the P-loop residues responsible for sodium selectivity into the TTX-binding site (Noda *et al.*, 1989; Terlau *et al.*, 1991), so perhaps the unusual arrangement of residues responsible for sodium-selectivity contributes to the unusual TTX-insensitivity in cnidarian channels.

The highly conserved inter-domain linker between domains III-IV is believed to play an essential role in fast inactivation of sodium channels (Catterall, 1996). This linker is 54 amino acids long in PpSCN1 and is the same size (53 or 54 amino acids) in most sodium channels. Three hydrophobic amino acids (IFM) are conserved in almost all

known channels, including the jellyfish channels, PpSCN1 and CYNA1 (Anderson *et al.*, 1993), (fig. 4-3B). The only exception in sodium channels is the sea anemone channel, AnemNa1 (White *et al.*, 1998). AnemNa1 has a phenylalanine (F) residue close to the appropriate position, but does not have an equivalent IFM motif and has a larger sized inter-domain linker (56 amino acids) preventing an unambiguous alignment of homologous residues (fig. 4-3B). It is believed that this region in sodium channels may fold over the open channel and stabilize the "lid" when it obstructs the open channel (Catterall, 1996). The receptor for this "lid" has been reported to include four, conserved hydrophobic residues (F, Y, V/I and I) in domain IV, segment 6 of sodium channels in higher phyla (McPhee *et al.*, 1995). This differs in cnidarian channels, with the exception of AnemNa1 (White *et al.* 1998) where F is replaced by V in jellyfish channels PPSCN1 and CYNA1 (Anderson *et al.*, 1993) and F by A in flatworms (highlighted residues, fig. 4-3b). The phenylalanine and tyrosine residues are critical for binding of the local anaesthetic lidocaine and for its characteristic use- and voltage-dependence (Ragsdale *et al.*, 1994). When a F1764A mutation is made in a rat brain channel, creating a flatworm-like sequence, use-dependent lidocaine block and inactivation were severely impaired (Ragsdale *et al.*, 1994). It will be interesting to determine if the sequence differences affect inactivation and lidocaine sensitivity in the flatworm and jellyfish channels when they are expressed *in vitro*.

In sodium channels, the fourth transmembrane segments have repeating positive charges that typically number 4, 5, 5 and 8 in domains I through IV respectively. Current

models have these charges acting as voltage-sensors, moving through the membrane in response to membrane depolarization and changing the conformation of the protein to open the channel pore (Yang *et al.*, 1996). All three cnidarian channels differ from this pattern by lacking the 5th charge of domain II (where Q replaces R/K) and by replacing positive charges in domain IV by a negatively charged residue, either E or D for R/K in the 6th residue in jellyfish channels PPSCN1 and CYNA1 (Anderson *et al.*, 1993) (fig. 4-4), or F for the 1st residue in AnemNa1 (White *et al.*, 1998). Variations from the "4-5-5-8" charge pattern in S4 are rare. Besides the cnidarian channels, only the channel cloned from squid optic lobe (Sato and Matsumoto, 1992) and a channel expressed in mammalian heart and uterus (SCN6A) (Felipe *et al.*, 1994; George *et al.*, 1992) show different patterns.

Mammalian brain and heart sodium channels are modulated by protein kinase A at sites in the cytoplasmic linker between I and II Cukierman, 1996), but none of these structural motifs are found in homologous positions in PpSCN1 (fig. 4-2) nor are they well conserved among mammalian channels. A highly conserved, modulatory site for protein kinase C site is located in the short, cytoplasmic linker between domains III and IV (Cukierman, 1996). The only channels not possessing this site are in cnidarians, PpSCN1 and CYNA1 (Anderson *et al.*, 1993), flatworm (Jeziorski *et al.*, 1997) and mammalian SCN6A (George *et al.*, 1992). The relative sparcity of modulatory sites for protein kinases in cnidarian channels like PpSCN1 in comparison with mammals and the

variability in number and placement of modulatory sites in mammals indicates that these sites were introduced under the selection pressures of individual channel types.

Diversity of sodium channels

Sodium channels appear to have evolved from calcium channels to allow high frequency signaling and rapid propagation (Hille, 1992) and appear to be far less diverse than either calcium or potassium channels. Calcium channels consist of at least 3 major metazoan classes (T-Type, L-type and non-L-type) that further subdivide in mammals into 7 different families (Stea *et al.*, 1995). Potassium channels are even more diverse, consisting of 3 metazoan structural classes, with one of the classes breaking down into 8 metazoan families, with multiple subfamily members in some families that come close to the total number of different mammalian sodium channel genes known to date (Wei *et al.*, 1996).

Nonetheless, sodium channels do show diversity in functional and pharmacological properties. For example, "slow" sub-threshold, sodium currents have been identified in Purkinje cells (French and Gage, 1985). Many voltage-gated sodium currents been characterized by their inactivation kinetics, ranging from very fast (Hille, 1992), to slow (Alonso and Llinas, 1989), to non-inactivating (Taylor, 1993). Sodium channels with different inactivation kinetics can influence action potential shape and play

significant physiological roles in mammals (Campbell, 1992). In *P. penicillatus*, at least three different sodium currents are present. There is a slowly activating and slowly inactivating sodium current in the electrically excitable epithelium, which is inhibited by high concentrations of internal calcium (Grigoriev and Spencer, 1996b). In contrast, "B" neurons in *P. penicillatus* have short duration, sodium-dependent action potentials (Spencer and Arkett, 1984), and the sodium current responsible for the action potential shows rapid inactivation and recovery from inactivation (<5 ms) (Grigoriev *et al.*, 1996a). The rapid inactivation recovery rate enables "B" neurons to fire at frequencies as high as 10 Hz (Spencer and Arkett, 1984). Lastly, there are sodium currents in swimming motor neurons (SMNs) which are similar to mammalian cardiac channels with rates of recovery from inactivation ~10 fold slower than typical mammalian neurons (Grigoriev *et al.*, 1996a). These properties have probably arisen by convergence since in both cases (SMNs and cardiac muscle) these cells act as pacemakers for fluid pumps (Fye, 1987).

In mammals, separate α subunit genes are responsible for the electrical and pharmacological differences between neuronal, skeletal muscle and cardiac sodium channels(Goldin, 1995). A least a dozen mammalian sodium channel genes are known, many of which have alternatively-spliced variants (Goldin, 1995). The properties of sodium channels in mammals are also modified by the presence of subunits and by phosphorylation (Adelman, 1995), thus creating ample structural and hence functional diversity. In comparison, no more than two pore-forming α subunit genes have been

detected so far from any single invertebrate species (cnidarians, nematodes, mollusks, insects). Even though jellyfish have functionally diverse sodium currents, PpSCN1 is the only sodium channel α subunit in *P. penicillatus* that we have detected. Only one ~6.0 kb mRNA transcript hybridized on Northern blots to a genomic probe that spanned the 5' end of the cDNA or a cDNA probe downstream, near the 3' end of the transcript (fig. 4-5). In addition, only a single copy of PpSCN1 is evident from Southern blots probed along the length of the gene with genomic fragments (see Chapter 5). No alternatively spliced variants appear to exist in PpSCN1, since the region spanning the full-length mRNA in genomic DNA was sequenced and no additional exons were found (see Chapter 5). However, the molecular techniques used to isolate PpSCN1 were designed to clone a sodium channel gene of close similarity to known channels, there may be other sodium-selective channel genes present in *P. penicillatus* with very different structures.

In mammals the kinetical properties of α subunits can be modified by co-expression with small accessory, ß subunits (Goldin, 1995). Full expression of the *Drosophila* α subunit requires an accessory subunit, TipE that is not homologous to the mammalian ß subunit (Feng *et al.*, 1995). We have not yet attempted to express PpSCN1, but it is probable that, like the other invertebrate α subunit clones (cnidarian (Anderson *et al.*, 1993; White *et al.*, 1998), flatworm (Jeziorski *et al.*, 1997) and molluscan (Dyer *et al.*, 1997; Rosenthal and Gilly, 1993; Sato and Matsumoto, 1992), it requires unique accessory subunits for expression.

None of the non-arthropod invertebrate genes (eg. Cnidarian, molluscan, flatworm) appear to express even in the presence of TipE from fly or the mammalian β subunits (Anderson and Dunn, 1997). It may be that some of the functional diversity of sodium channels in invertebrates may be accounted for by the presence of accessory subunits and/or post-translational modification of α subunits and that α subunit diversity is limited.

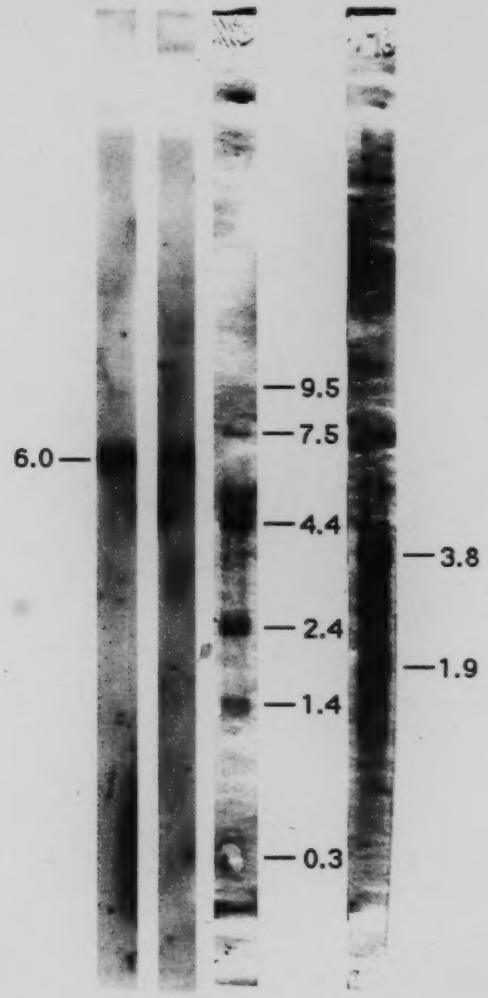

Figure 4-4. PpSCN1 identified in *P. penicillatus* polyadenylated mRNA using Northern blot hybridization analysis. ^{32}P-labeled probes were a genomic fragment (lane 1) and a cDNA fragment (lane 2). Methylene blue stained standards (lane 3) and *P. penicillatus* total RNA (lane 4). Total RNA in lane 4 appears undegraded with ribosomal bands at 3.8 and 1.0 kb. The cDNA probe is near the 3' end (illustrated in fig. 4-1). The genomic probe is 6.41 kb and spans the cDNA from positions 477 to 2224 at the 5' end of the gene.

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CHAPTER 5 GENOMIC ORGANIZATION OF A VOLTAGE-GATED Na^+ CHANNEL (PPSCN1) IN A HYDROZOAN JELLYFISH[10,11]: IMPLICATIONS FOR THE STRUCTURAL EVOLUTION OF VOLTAGE-GATED Na^+ AND Ca^{2+} CHANNELS

INTRODUCTION

The simplest extant eumetazoans with a discrete nervous system are cnidarians, which have only two germ layers (diploblastic). Extant cnidarians are structurally very similar to fossilized cnidarians in the Ediacarian fauna, suggesting that the genes responsible for physiological functions are also likely to be reminiscent of an ancestral phenotype. In the hydrozoan jellyfish, *Polyorchis penicillatus*, "swim motor neurons" have rapid, transient Na^+-selective currents that are kinetically and electrically like Na^+ currents in mammals (Grigoriev *et al.*, 1996). However, they are insensitive to classical

[10] A version of this chapter has been submitted for publication. Spafford, J.D., Spencer, A.N. and Gallin, W.J. (1998) *Mol.Biol.Evol.*

[11] The nucleotide and deduced amino acid sequences reported in this paper have been deposited in GenBank (Accession # AF047379). The published GenBank script for PpSCN1 is shown in Appendix B.

Na^+ channel agonists and antagonists, including tetrodotoxin, even at high concentrations (100 μM) (see Chapter 2).

We have recently determined the sequence of a full-length cDNA of a Na^+ channel α subunit (PpSCN1) isolated from this jellyfish, coding for a 1695 amino acid protein (see Chapter 4). It has the typical topology of Na^+ channels, consisting of four homologous domains, each domain containing six transmembrane segments.

We have sequenced the complete genomic region spanning the cDNA of PpSCN1 and present here a detailed analysis of our findings. Na^+ channel genes share closely related homologous sequences, from jellyfish to mammals. Alignments of these genes underscore possible structural-functional similarities and differences. Given the long evolutionary history that separates cnidarian and mammalian channel genes, it provides an opportunity to evaluate the tolerance of structural features to evolutionary change, and hence to adaptive structural changes taken by different channel types. We show that most of the introns are conserved between Na^+ channel genes in *P. penicillatus* and mammals, with a significant percentage even shared with Ca^{2+} channels. The conservation extends to the details of intron splice sites, including a recently discovered U12-type splice site that employs a rarely used set of small nuclear ribonucleoproteins. Such conservation places the intron splicing mechanisms and the primary exon arrangement of Na^+ channels to at least the common ancestors of diploblasts and triploblasts, ~600 million to 1 billion years ago (Morris, 1993).

MATERIALS AND METHODS

Genomic DNA Isolation and Construction of Libraries

Genomic DNA was isolated from a pool of gonads dissected from approximately 200 adult medusae of *Polyorchis penicillatus*. The material was rapidly frozen and stored at –80°C until extraction. The frozen tissue was then ground to a powder in liquid nitrogen using a mortar and pestle and the powder was resuspended in a Proteinase K/SDS extraction buffer (Ausubel *et al.*, 1997) and digested at 50°C for 6 hours. The solution was then extracted several times with phenol and chloroform. DNA was precipitated from the aqueous phase with 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of ethanol. The DNA was spooled out, washed twice with 70% ethanol, dried briefly under vacuum and redissolved in 20 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

To prepare DNA for constructing libraries, 100 µg aliquots of DNA were digested in 500 µl reactions with 5 units of Sau3A for 5 and 10 minute periods. At the end of the incubation the reactions were stopped by adding EDTA. The reactions were pooled and resolved on a 0.3% agarose gel. DNA between 15 and 30 kb was excised from the gel and harvested using a GeneClean II kit (Bio 101, La Jolla). The size-selected DNA was then dephosphorylated with alkaline phosphatase (Ausubel *et al.*, 1997) to avoid double insertion. The insert material was ligated into BamHI-cut, EMBL3 arms (Stratagene, La

Jolla) and the ligations were packaged using GigaPack Gold reactions (Stratagene, La Jolla). The resulting phage were titrated and then amplified to yield four independent libraries, each with a complexity of $2X10^5$.

Isolation of PpSCN1 genomic clones

Fragments of the Na^+ channel gene PPSCN1 were isolated from *P. penicillatus* genomic DNA by PCR using degenerate and inverse PCR primers (details described in Chapter 4). A 941 bp PCR product (probe B, fig. 5-1) was ^{32}P-labeled and used to isolate a full-length cDNA (GenBank Accession # AF047380, described in Chapter 4) and genomic clones PpSCN1.1g and PpSCN1.2g.

DNA probes were ^{32}P-labeled by incorporation of radioactive nucleotides in the synthesis of DNA in PCR or labeled with random hexamers and the large fragment of DNA polymerase I (Klenow). Duplicate Hybond-N nylon filters (Amersham, Little Chalfont) were lifted from plates with 5×10^4 plaque-forming units per plate. Hybridization was carried out using the method of Church and Gilbert (Church and Gilbert, 1984), performed at 65°C in 0.5 M Na^+, no formamide and washing was performed at 65°C in 40 mM Na^+.

Southern blot analysis, subcloning and DNA sequencing

EcoRI-cut genomic fragments (probe A, C to E, fig. 5-1) and probe B were ^{32}P-labeled and used to probe a Southern blot of genomic DNA (5 µg of DNA per lane) from P. *penicillatus*. DNA probes were ^{32}P-labeled by incorporation of radioactive nucleotides in the synthesis of DNA in PCR or labeled with random hexamers and the large fragment of DNA polymerase I (Klenow). Southern blots were prepared using a standard protocol (Ausubel *et al.*, 1997) and a Genescreen Plus nylon hybridization transfer membrane (Dupont NEN, Boston) with hybridization conditions as for library screening using the method of (Church and Gilbert, 1984). Genomic DNA and PCR fragments were digested with restriction enzymes, subcloned into pBluescript KS+ vector (Stratagene, La Jolla) and sequenced in both directions with a Perkin-Elmer ABI 373A sequencer using an ABI Prism Dye-Terminator Cycle Sequencing Kit. PCR products and gel-extracted DNA were purified using silica resin (Hengen, 1995) with an additional purification for sequencing through QIAGEN-tip 20 columns (QIAGEN, Chatsworth). Exon/intron boundaries were determined by comparing the genomic sequences with the corresponding Na^+ channel cDNA sequence (PpSCN1) and identifying consensus acceptor and donor sites in the genomic sequence.

Analysis of DNA and amino-acid sequences

MacVector 6.0 (Oxford Molecular Group), Gene Construction Kit 2 (Textco, Inc.) and GCG v8.1 (Genetics Computer Group, 1995) were used for alignments, assembly and analysis of DNA and amino-acid sequences. Sequences for other proteins were retrieved in the GenBank database using BLAST (Altschul *et al.*, 1990). Multiple alignments were made using a modified progressive pair-wise alignment method of Feng and Doolittle (Feng and Doolittle, 1987) in PileUp (GCG, 1995). Gap creation and extension penalties were 3.0 and 0.10 respectively. Aligned amino acid sequences were imported into PAUP v3.1.1 (Swofford, 1991) to generate the most parsimonious gene tree. Regions of low sequence similarity between Ca^{2+} and Na^+ channels were not included in the data matrix. These regions are the amino and carboxyl termini, the cytoplasmic loops and the extracellular loop between segments 5 and 6 in domain I. Minimum length trees were found using a heuristic search method with 25 repeats. Each character state change was scored using a step matrix that reflected the minimum number of nucleotide changes that were required for a given change in amino acid. Statistical support for the tree was evaluated by 100 replicate bootstraps (Felsenstein, 1985) as implemented in PAUP. The phylogenetic tree was displayed using TreeView 1.5 (Page, 1996). PlotSimilarity (Genetics Computer Group 1995) was used to plot the running average of the similarity among aligned Na^+ channel sequences from PileUp.

RESULTS

Cloning and description of PpSCN1

A 941 bp PCR product (probe B, fig. 5-1) was used to isolate two genomic fragments, PpSCN1.1g (13.5 kb) and PpSCN1.2g (28 kb) from genomic libraries by high-stringency hybridization screening. The complete 13,941 bp region spanning the cDNA was sequenced from genomic clone PpSCN1.2g and compared to the cDNA (fig. 5-1, top). The clone covers the full-length 5774 bp mRNA transcript, which has a 5085 bp open reading frame coding for a 1695 amino-acid protein. No alternative exons were found in the gene. Sequencing of part of the coding region of PpSCN1.1g and analysis of restriction digest maps confirms that both genomic clones probably represent the same gene. At least 15 synonymous differences are present in the coding regions of PpSCN1. These differences are likely due to polymorphisms since tissues were pooled from many jellyfish to construct the cDNA and genomic libraries.

Only the expected gene, PpSCN1 was clearly discernible on Southern blots probed at high stringency with DNA fragments (A-E) that cover most of the coding region of the Na^+ channel gene (fig. 5-1). Other bands in Southern blots of fig. 5-1 were faint, inconsistent, and have high molecular weights, indicative of incomplete digestion.

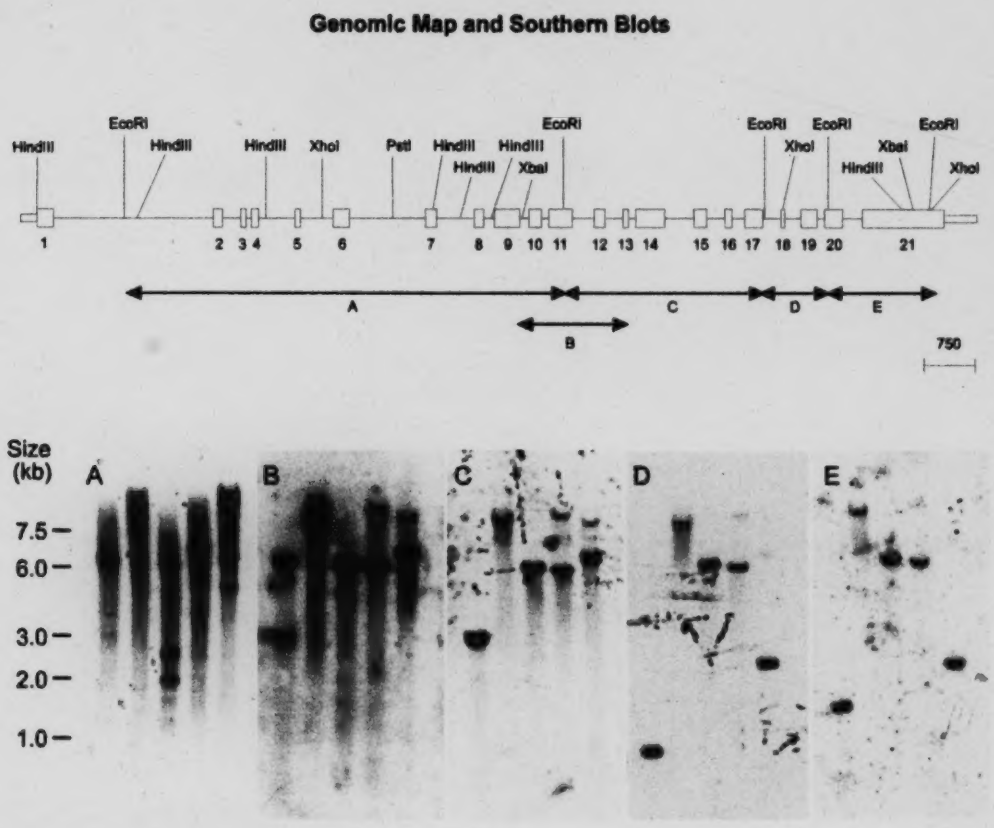

Figure 5-1. Restriction map illustrating intron / exon structure (top) and Southern blot analyses (bottom) of PpSCN1 gene. Exons are numbered 1 to 21 and represented by open boxes; the 5' and 3' untranslated regions are indicated by thinner open boxes. Lines with tipped arrows span genomic fragments A to E used to probe Southern blots. Each S. blot contains 5 lanes of genomic DNA from 5 μg of *P. penicillatus* DNA per lane digested with EcoRI, PstI, HindIII, XbaI, XhoI, respectively.

The coding region of PpSCN1 consists of 21 exons, ranging from 54 bp to 1656 bp in length for an average of 275 bp (boxes in map of fig. 5-1). 20 intervening sequences separate the exons and they range in size from 78 bp to 2332 bp, with an average of 408 bp (lines separating boxes in map of fig. 5-1).

Phylogenetic comparisons

The most parsimonious phylogenetic tree based on aligned amino acids of genes from the major subdivisions within the Na^+ and Ca^{2+} superfamily is illustrated in fig. 5-2, with bootstrap values shown at the branch nodes. According to the tree, Na^+ channels must have evolved very early in metazoan history, from ancestors resembling the T-type more than the non-L or L-type Ca^{2+} channels. The divergence of Na^+ channels likely occurred before the separation of the protostomes and deuterostomes, since the L, non-L and T-type Ca^{2+} channels are clearly found in the protostomes (eg. nematodes and arthropods) and the deuterostomes, and the single cnidarian Ca^{2+} channel is included within the L-type subclass and not basal to both L-type and non-L type subclasses.

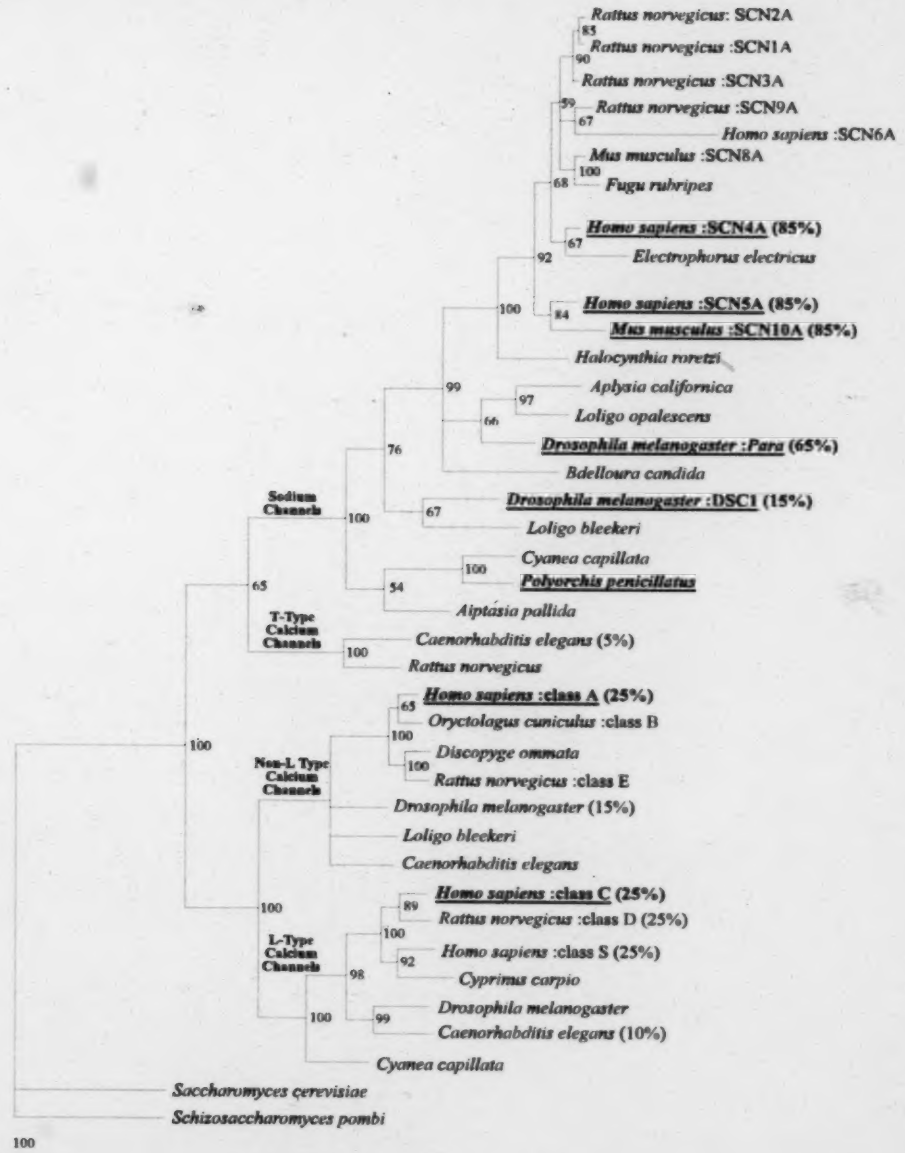

Figure 5-2. Phylogenetic tree of selected voltage-gated Ca^{2+} and Na^+ channels generated using maximum parsimony. Bootstrap values for each branch point are shown. Genes that are underlined are described in Table 5-1. The percentage of intron sites shared with PpSCN1 are shown after those genes in which the intron-exon structures have been determined.

Together PpSCN1, from the hydrozoan jellyfish *Polyorchis penicillatus*, and CYNA1, a scyphozoan jellyfish (Anderson *et al.*, 1993) form a deeply branched, two gene subgroup within the Na^+ channel family (fig. 5-2), with an overall match in amino-acid similarity / identity of 77.4% / 62.8%. More distantly related to PpSCN1 is an anthozoan gene (AnemNa1) (White *et al.*, 1998) with an overall match in similarity / identity to PpSCN1 of 65.8% / 47.0%.

The out-group for the gene tree (fig. 5-2) consists of voltage-gated cation channels from two yeast species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombi* (GenBank Accession #s: X87941, Z98981, respectively). The yeast genes share a close similarity with metazoan Na^+ and Ca^{2+} channel classes having a cation channel structure with four domains. They have homologous residues that line the selectivity filter that, based on sequence, likely provide Ca^{2+}-selectivity or selectivity for Na^+ and Ca^{2+} ions (Sather *et al.*, 1994). The tree contains the following animal genes, (with their common names, accession numbers): **Na^+ channels**: SCN1A (rat, X03638), SCN2A (rat, X03639), SCN3A (rat, Y00766), SCN4A (human, M81758), SCN5A (human, M77235), SCN6A (human, M91556), SCN8A (mouse, U26707), SCN9A (rat, AF000368), SCN10A (mouse, Y09108), *Fugu rubipes* (puffer fish, D37977), *Electrophorus electricus* (electric eel, X011119), *Halocynthia roretzi* (ascidian, D17311), *Loligo opalescens* (squid, L19979), *Loligo bleekeri* (squid, D14525), *Aplysia californica* (sea hare, U66915), *Para* (fruit fly, M32078), DSC1 (fruit fly, Y00461), *Bdelloura candida* (flatworm, U93074), *Aiptasia pallida* (sea anemone, AF041851), *Cyanea capillata*

(scyphozoan jellyfish, L15445), *Polyorchis penicillatus* (hydrozoan jellyfish, AF047380); **T-type Ca^{2+} channels**: *Rattus norvegicus* (rat, AF027984) *Caenorhabditis elegans* (nematode, U37548); **non-L type Ca^{2+} channels**: class A (human, X99897), class B (rabbit, D14157), class E (rat, L15453), *Discopyge ommata* (marine ray, L12531), *Loligo bleekeri* (squid, D86600), *Drosophila melanogaster* (fruit fly, U55776), *Caenorhabditis elegans* (nematode, U25119); **L-type Ca^{2+} channels**: class C, (human, L29529), class D (rat, D38101), class S (human, L33798), *Cyprinus carpio* (carp, M62554), *Drosophila melanogaster* (fruit fly, U00690), *Caenorhabditis elegans* (nematode, AF023602), *Cyanea capillata* (scyphozoan jellyfish, U93075).

Genomic structure of PpSCN1 compared to other cation channel genes

Genomic structures of PpSCN1 and other Na^+ and Ca^{2+} channels are compared in Table 5-1 (underlined in the phylogenetic tree, fig. 5-2). Since intron positions may appear close together by chance without having a common origin, a shared intron position is defined in the strictest sense: It separates the coding region in the identical phase of the codon of aligned amino-acids in a region of high homology.

Table 1. General Description of Genomic Structures of Ca^{2+} and Na^+ Channel Genes

Channel Description	GenBank Identifier	Ion selectivity	Size of Coding Region (amino acids)	Size of Coding Region Spanning in Genomic DNA (bp)	# of Invariant Exons	# of Alternate / Optional Exons	# of Introns	# and (%) of Intron Sites Shared with PpSCN1
hydrozoan jellyfish	PpSCN1	Na^+	1695	13.1	21	0	20	
fruit fly (*Drosophila*)	*Para*	Na^+	2157	60+	17+	7	16+	13+ (65%+)
fruit fly (*Drosophila*)	DSC1	Na^+	1622+	n.r.*	12+	n.r.*	11+	3+ (15%+)
human skeletal	SCN4A	Na^+	2016	32.5	24	n.r.*	23	17 (85%)
human heart	SCN5A	Na^+	1836	80	27	n.r.*	26	17 (85%)
mouse sensory	SCN10A	Na^+	1958	90	27	n.r.*	26	17 (85%)
human L-type	CACNLTA1	Ca^{2+}	1651	150	44	6	43	5 (25%)
human non-L type	CACNL1A4	Ca^{2+}	2261	300	47	n.r.*	46	5 (25%)

*none reported

An alignment of amino acid sequences of PpSCN1 with a representative Na^+ channel, the TTX-sensitive human skeletal muscle (SCN4A) (George *et al.*, 1993; McClatchey *et al.*, 1992) and a representative Ca^{2+} channel, human L-type, Class C, Ca^{2+} channel from human fibroblasts (CACNL1A1) (Soldatov, 1994), is shown in fig. 5-3, with intron positions indicated by a bar. A closer examination of the aligned, homologous DNA sequences surrounding the intron splice is described in Table 5-2. Eighty-five percent of intron splice junctions in PpSCN1 are found in mammalian Na^+ channels. Most of the same locations of introns were also reported for a Na^+ channel gene expressed in brain and spinal cord (SCN8A) (Plummer *et al.*, 1997). In *Drosophila melanogaster*, 15% and 65% of the known intron sites in DSC1 and *Para* are found in the homologous position in PpSCN1, although the full genomic structure is not known for either of these fly genes (Loughney *et al.*, 1989; Salkoff *et al.*, 1987). The intron positions are highly conserved in Na^+ channels from jellyfish to mammals, in spite of the lack of similarity of intron sequences and large difference in intron sizes among the Na^+ channel genes. The average intron sizes in the Na^+ channel genes listed in Table 5-1 are ~2.7 to ~7.7 times greater than in PpSCN1.

Since Na^+ channels are considered to be derived from Ca^{2+} channels, it might be expected that there would be some vestige of their common history in their genomic structures. Five introns shared among jellyfish and mammals Na^+ channel genes are also found in mammalian L and non-L type Ca^{2+} channels (fig. 5-3, Table 5-1).

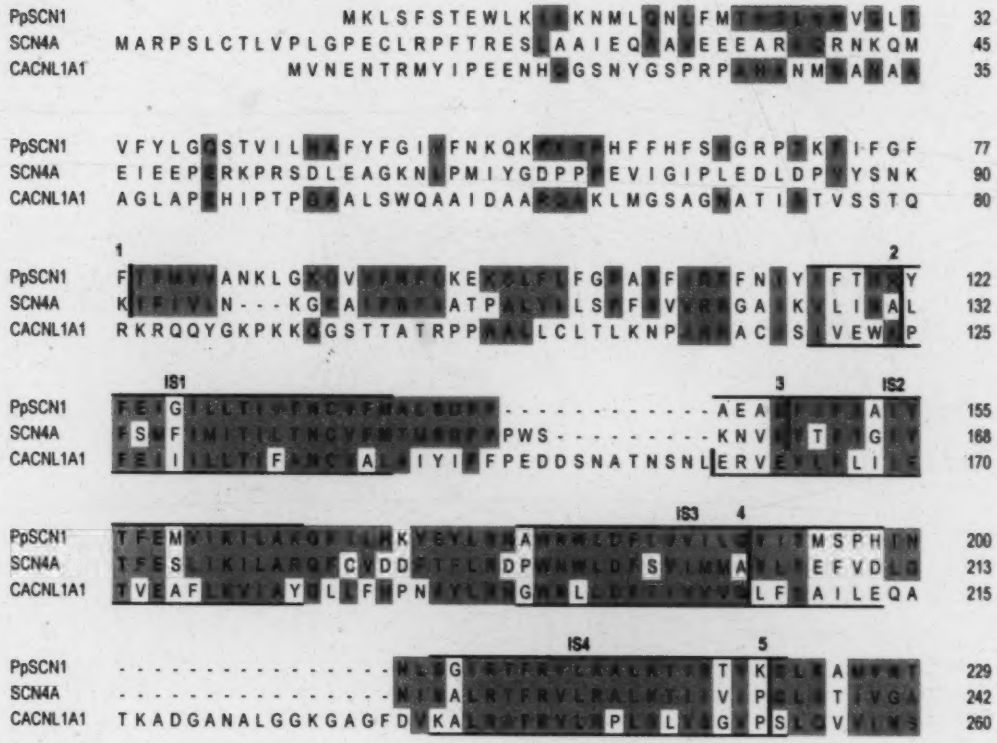

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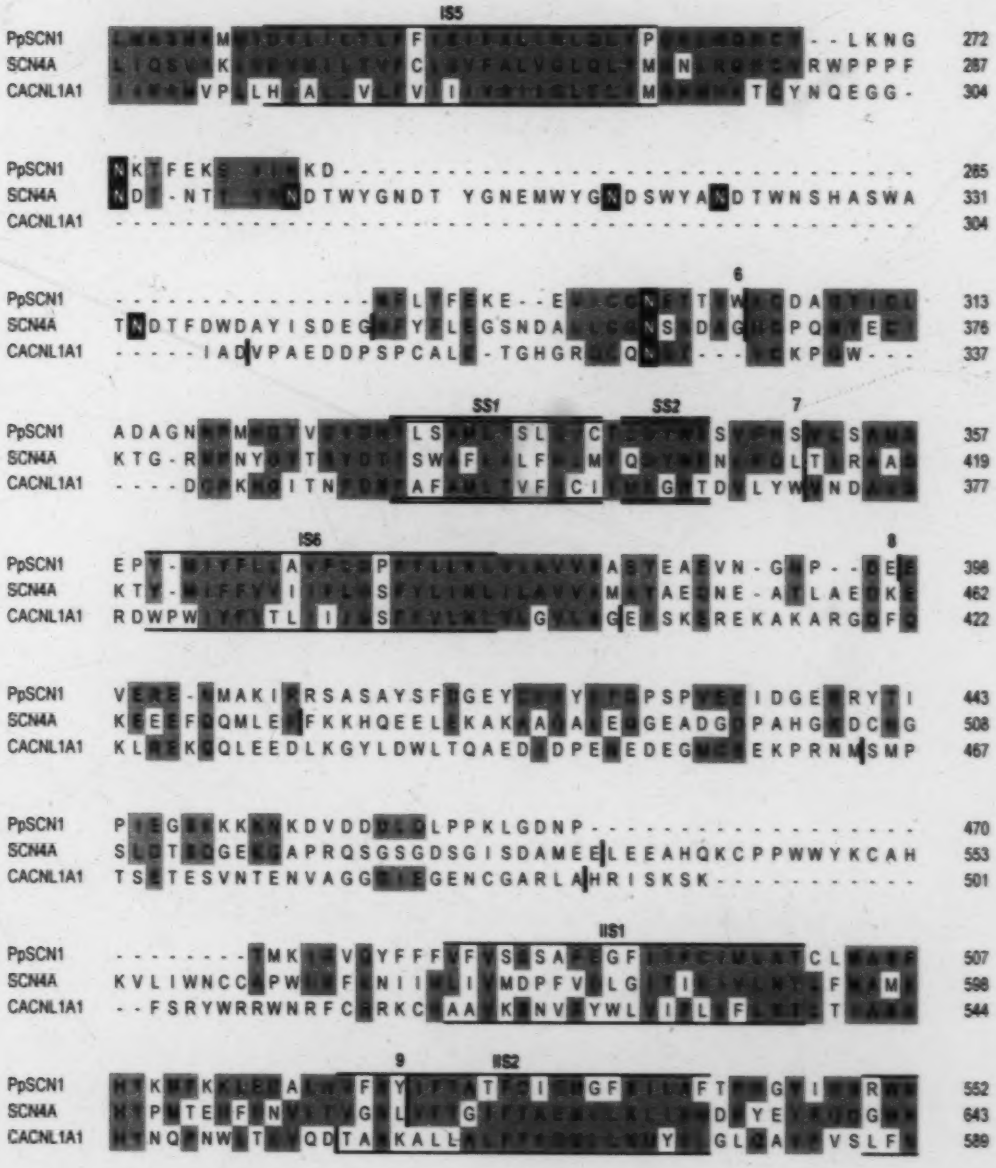
Figure 5-3. Intron splice junctions (vertical bars) in the aligned amino-acid sequences of PpSCN1, human skeletal muscle Na^+ channel (SCN4A) and a human L-type Ca^{2+} channel (CACNL1A1) genes. Intron splice sites for PpSCN1 are labeled 1 to 20 and shown as described for SCN4A (McClatchey et al., 1992; George et al., 1993) from accession numbers L04216 to L04236 and for CACNL1A1 (Soldatov, 1994) from accession numbers Z26256 to Z26287. Single lined, top and bottom borders surround the residues for the putative transmembrane segments 1 to 6 in domains 1 through 4. Short segments 1 and 2 (*SS1* and *SS2*) are indicated by double lined top and bottom borders. Putative N-type glycosylation sites for the extracellular loop in D1 and D3 are indicated by inverted residues. Similar amino-acid sequences are shaded.



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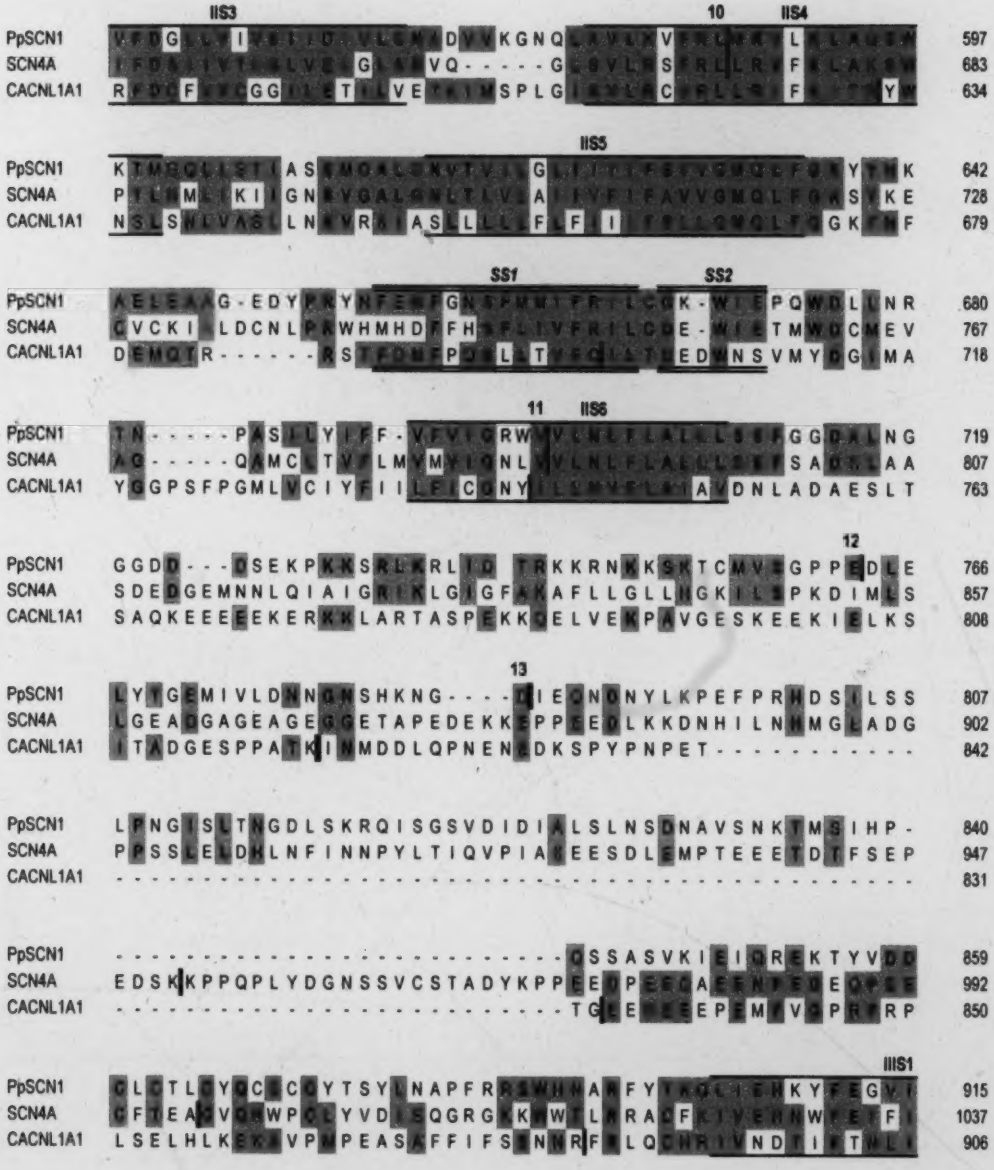

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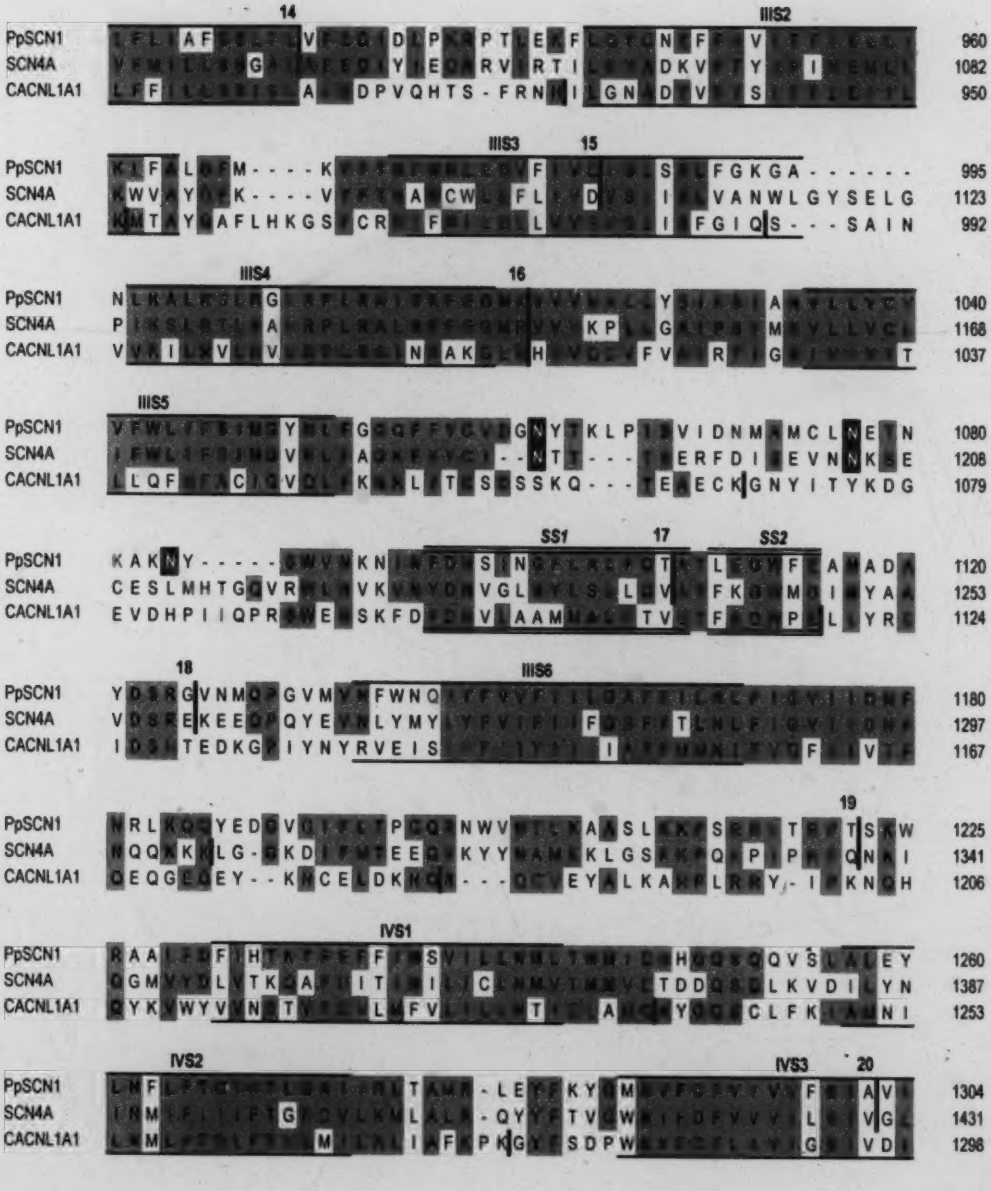

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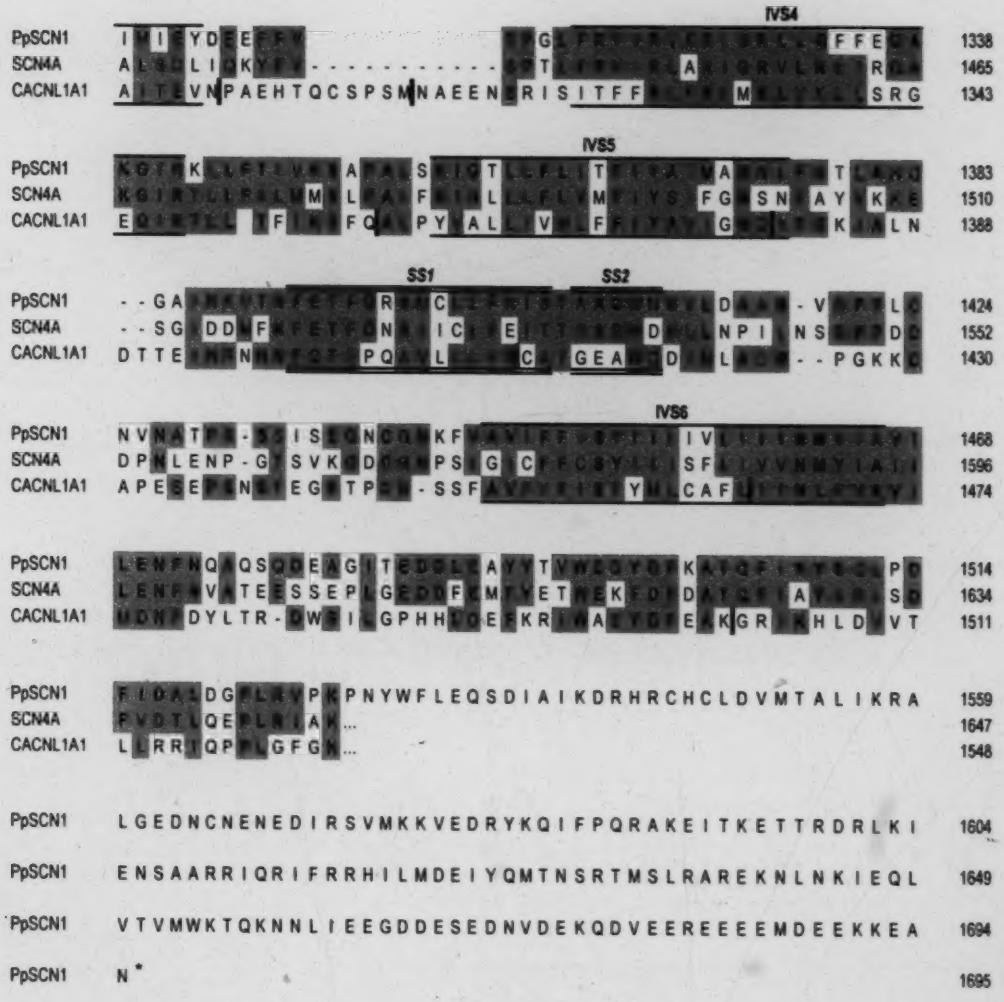
IVS4
PpSCN1
SCN4A
CACNL1A1
1338
1465
1343
IVS5
PpSCN1
SCN4A
CACNL1A1
1383
1510
1388
SS1
SS2
PpSCN1
SCN4A
CACNL1A1
1424
1552
1430
IVS6
PpSCN1
SCN4A
CACNL1A1
1468
1596
1474
PpSCN1
SCN4A
CACNL1A1
1514
1634
1511
PpSCN1
SCN4A
CACNL1A1
1559
1647
1548
PpSCN1
1604
PpSCN1
1649
PpSCN1
1694
PpSCN1
1695

Table 5-2

Catalogue of Intron Splice Sites in PpSCN1 and Shared Sites in a Representative Na^+ Channel (SCN4A) and a Calcium Channel (CACNL1A1)

Channel #	Name	Exon Size	Donor site Exon | Intron...	Acceptor site ...Intron | Exon	Intron size
U2-Type	(GT-AG)	consensus:	|GTRAGT..	..YYNYAG|	
1	PpSCN1	475	GGA TTT TTC | GTTACA..	..TTTTAG | ACA TTT ATG	2332
			G F F	T F M	
	SCN4A		AAT AAG AAG | GTCTGG..	..CCTTAG | ACC TTC ATC	
			N K K	T F I	
*2	PpSCN1	128	ACT CAT CA |	| A TAT TTT GAA	261
			T H R	Y F E	
	SCN4A		ATC CAT GC |	| G CTG TTC AGC	
			I H A	| L F S	
	CACNL1A1		GAA TGG AA |	| A CCA TTT GAA	
			E W K	P F E	
3	PpSCN1	80	GAA GCA GA | GTAAGT..	..TTTTAG | A TTT ATT TTT	78
			E A E	F I F	
	SCN4A		AAT GTG GA | GTAAGT..	..CCCCAG | G TAC ACC TTC	
			N V E	Y T F	
*4	PpSCN1	128	ATA CTA GG | GTAAGA..	..GTTTAG | G TAT ATT ACC	505
			I L G	Y I T	
	SCN4A		ATG ATG GC | GTGACG..	..ACCCAG | G TAC CTG ACA	
			M M A	Y L T	
	CACNL1A1		GTT GTT GG | GTAAGT..	..TTCTAG | G CTT TTT AGT	
			V V G	L F S	
*5	PpSCN1	91	ACT GTC AAA G | GTAAAC..	..TTTAAG | GT TTA AAA	469
			T V K	G L K	
	SCN4A		GTC ATC CCA G | GTACTG..	..GGTCAG | GG CTG AAG	
			V I P	G L K	
	CACNL1A1		GGA GTC CCA A | GTAAGT..	..CCGCAG | GT CTC CAG	
			G V P	S L Q	

*Intron Sites shared in Na^+ and Ca^{2+} channels

- Table 5-2 continues on next page –

#	Channel Name	Exon Size	Donor site Exon|Intron	Acceptor site Intron|Exon	Intron size
	U2-Type (GT-AG)	consensus:	|GTRAGT	..YYNYAG|	
6	PpSCN1	245	ACG TCA TG|GTAAAT	..TATTAG|G AAA TGT GAC	1115
			T S W	K C D	
	SCN4A		GAT GCT GG|GTAAGA	..TCCTAG|G CAC TGC CCT	
			D A G	H C P	
*7	**PpSCN1**	**114**	**TTT AAC AGT|GTAAGT**	**..ATTTAG|GTC CTT TCA**	**572**
			F N S	**V L S**	
	SCN4A		**TTC CAG CTG|GTACAG**	**..TCCCAG|ACC CTT CGA**	
			F Q L	**T L R**	
	CACNL1A1		**CTG TAC TGG|GTACGT**	**..TCTCAG|GTC AAT GAT**	
			L Y W	**V N D**	
8	PpSCN1	138	CCA GAT GAA|GTAAAT	..TTTAAG|GAA GTG GAA	156
			P D E	E V E	
9	PpSCN1	378	TTT AAT TAT|GTAAGA	..TTCCAG|ATA TTT ACA	111
			F N Y	I F T	
	SCN4A		GGC AAC CTC|GTAGGG	..CCCTAG|GTC TTC ACA	
			G N L	V F T	
10	PpSCN1	189	TTT CGC TTA|GTAAGA	..TTATAG|ATG CGG GTA	109
			F R L	M R V	
	SCN4A		TTC CGT CTG|GTAGGG	..TGGCAG|CTG CGG GTC	
			F R L	L R V	
11	PpSCN1	351	AGA TGG GTG|GTAAGT	..TTTCAG|GTA CTC AAT	301
			R W V	V L N	
	SCN4A		AAT CTT GTG|GTGAGT	..CCCCAG|GTC CTG AAC	
			N L V	V L N	
12	PpSCN1	177	CCA CCC GAA|GTACGT	..TTATAG|GAT CTG GAA	267
			P P E	D L E	
13	PpSCN1	70	AAT GGT GAC A|GTAAGA	..CTACAG|TT GAA CAA	115
			N G D	I E Q	

*Intron Sites shared in Na^+ and Ca^{2+} channels

- Table 5-2 continues on next page –

#	Channel Name	Exon Size	Donor site Exon|Intron..	Acceptor site ..Intron|Exon	Intron size
	U2-Type (GT-AG)		consensus: |GTRAGT..	..YYNYAG|	
14	PpSCN1	417	TTG ACC TTA|GTAAGT..	..TTATAG|GTA TTT GAA	431
			L T L	V F E	
	SCN4A		GGG GCT CTG|GTAGGC..	..CCCCAG|GCC TTC GAG	
			G A L	A F E	
15	PpSCN1	174	ATT GTG TGT|GTAAGT..	..TTGCAG|ATT TCA TTG	266
			I V C	I S L	
	SCN4A		ATC GTG GAT|GTGAGT..	..CTACAG|GTC TCC ATC	
			I V D	V S I	
***16**	**PpSCN1**	**105**	**GGA ATG AAA|GTAAGT..**	**..TTCCAG|GTG GTC GTC**	**179**
			G M K	**V V V**	
	SCN4A		**GGC ATG AGG|GTGGTG..**	**..CCCCAG|GTG GTG GTG**	
			G M R	**V V V**	
	CACNL1A1		**GGG CTA AAG|GTGAGT..**	**..CTGCAG|CAT GTG GTT**	
			G L K	**H V V**	
17	PpSCN1	279	TTT CAA ACG|GTAATT..	..CTACAG|GCG ACA TTG	247
			F Q T	A T L	
	SCN4A		CTG CAG GTG|GTGAGT..	..TCTCAG|GCC ACC TTC	
			L Q V	A T F	
18	PpSCN1	54	TCG AGA GGT|GTAAGT..	..TATTAG|GTT AAC ATG	246
			S R G	V N M	
	SCN4A		TCC CGG GAG|GTGAGT..	..CCACAG|AAG GAG GAG	
			S R E	K E E	
19	PpSCN1	246	AGG CCT ACG|GTAATG..	..TTTCAG|AGC AAA TGG	107
			R P T	S K W	
	SCN4A		CGG CCC CAG|GTACAG..	..CTGCAG|AAC AAG ATC	
			R P Q	N K I	
20	PpSCN1	271	TCG ATT GCA G|GTATGT..	..TTTTAG|TG ATA ATA	292
			S I A	V I I	
	SCN4A		TCC ATT GTG G|GTGAGC..	..TCGCAG|GC CTT GCC	
			S I V	G L A	
21	PpSCN1	1656	GAA GCT AAC taa… 447 bp 3′ UTR		
			E A N *		

***Intron Sites shared in Na^+ and Ca^{2+} channels**

Intron positions relative to the protein sequence

All the conserved introns between mammals and jellyfish occur in the major membrane spanning domains I through IV and the short, inter-domain linker between domains III and IV (ID3-4), which are regions of highest sequence similarity between Na^+ channels (fig. 5-4). In fig. 5-5, the intron locations are superimposed on the predicted membrane topology (shown to scale) of PpSCN1 and representative Na^+ channels. The intron locations that are unique to mammals or to each channel species (PpSCN1, *Para*, SCN4A, SCN5A) are located primarily in the regions of the greatest size and sequence differences between PpSCN1 and other Na^+ channel genes. These intron locations are in the cytoplasmic linkers between domains ID1-2 and ID2-3 and the extracellular loop between segment 5 and the membrane-penetrating, pore-forming P segment (short-segments I and II, fig. 5-5) between segment 5 and segment 6 in domain I (D1/S5-P). An intron located near the 5' end of ID3-4 (fig. 5-4), is the only intron present in a conserved region in mammalian genes not found in PpSCN1. Although the intron is not found in *Para* from *D. melanogaster* as well, it is present in a Na^+ channel of the same species (DSC1) (Salkoff *et al.*, 1987) and in a Na^+ channel from the sea hare, *Aplysia californica* (Dietrich *et al.*, 1997).

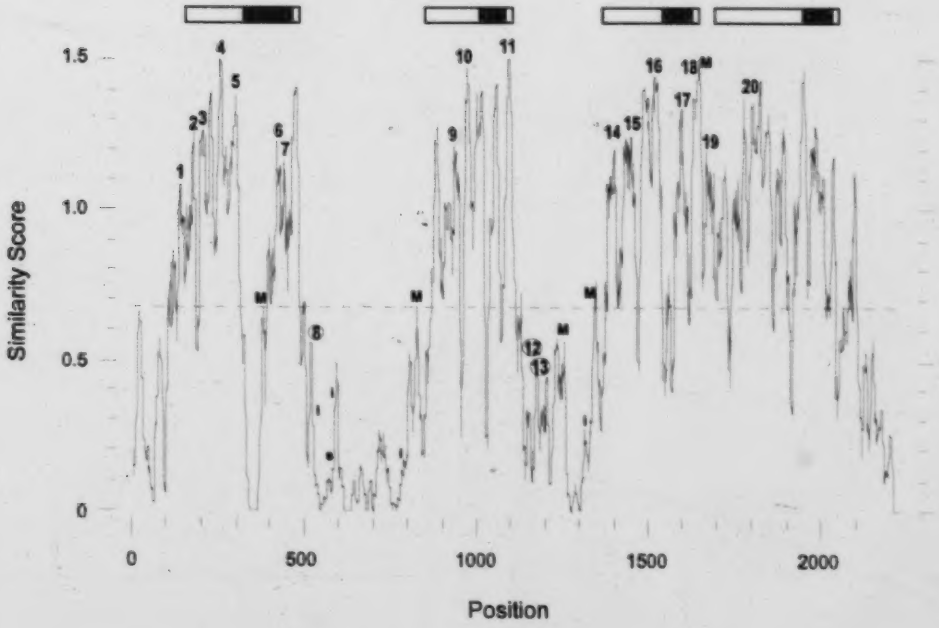

Figure 5-4. Running average of similarity among aligned amino acids of Na^+ channel genes, PpSCN1 (hydrozoan jellyfish), SCN4A (human skeletal muscle) and SCN5A (human heart). Intron sites for PpSCN1 are labeled from 1 to 20; Intron numbers 8, 12 and 13 are unique to PpSCN1. The one intron site unique to SCN4A is labeled with (**s**), the four unique intron sites for SCN5A are labeled with (**i**) and the shared intron sites unique among mammalian genes (SCN4A, SCN5A, SCN10A) are labeled with (**M**). The sliding window of comparison for the plot was 10 bp. Similarity scores of 1.5 are perfect matches and values less than 1.5 (depending upon the evolutionary distance) for non-matches. The four membrane spanning domains are indicated by boxes, with the extracellular loop and pore-lining region between segments 5 and 6 indicated by dark boxes.

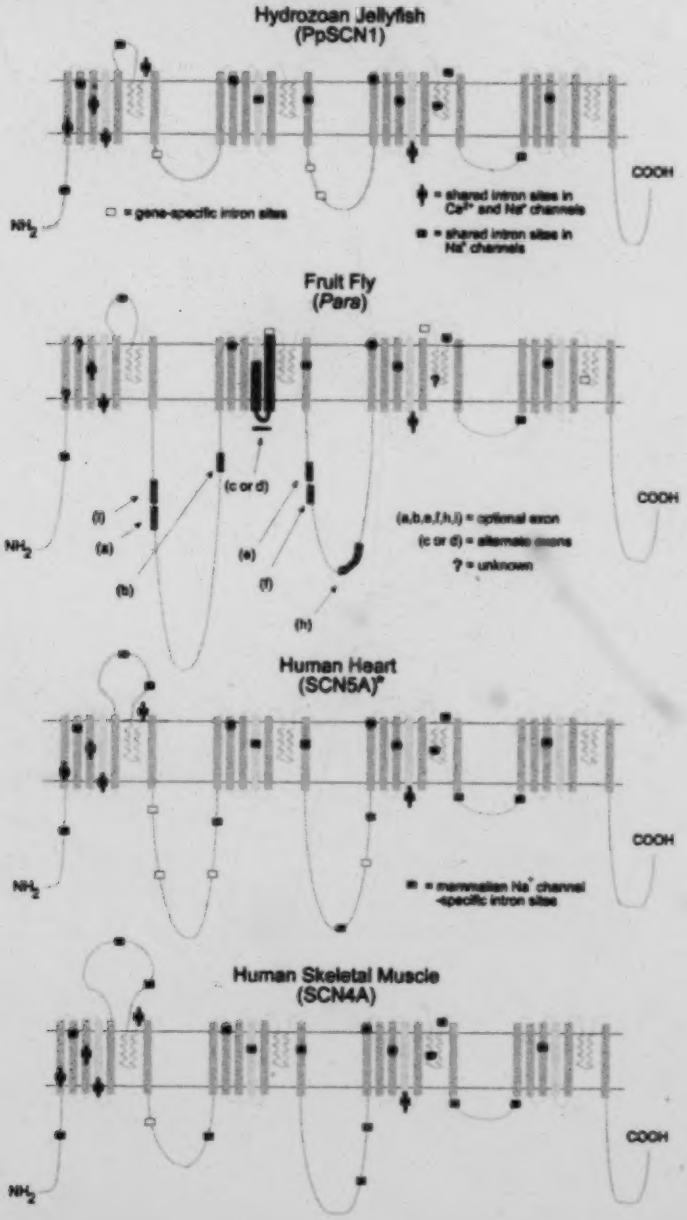

Figure 5-5. Locations of introns (small boxes) in relation to the membrane topology of Na^+ channel genes PpSCN1 (hydrozoan jellyfish), *Para* (fruit fly), SCN4A (human skeletal muscle) and SCN5A (human heart). Longer, shaded boxes in the fruit fly gene *Para* indicate regions where alternative and optional exons are located. *Intron splice junctions that are shared between SCN4A and SCN5A are conserved in SCN10A, isolated from mouse sensory neurons.

SCN10, isolated from mouse sensory neurons, is closely related to SCN5A, as shown by, the gene tree of fig. 5-2, the same number of introns and the similar arrangement of intron splice sites (fig. 5-4). The slight differences in location of introns between SCN10A and SCN5A are due to the same variably located introns that differentiate SCN5A from SCN4A.

In fig. 5-6, an alignment of the extracellular loop in domain I (D1/S5-P) is shown with the corresponding homologous region in domain III (D3/S5-P) for jellyfish and mammalian channels, PpSCN1, SCN4A and SCN5A, respectively. S5-P is bordered by glycosylation sites (labeled A and B, fig. 5-6), that are in similar positions in almost all Na^+ channels in D1 and D3. C-terminal from B is another conserved glycosylation site in D3 of many Na^+ channels, site C (although not present in SCN4A, fig. 5-6). Site B in D1 is conserved in the homologous position in most L and non-L type Ca^{2+} channels as well (see positions of CACNL1A1 in fig. 5-3). The divergence of the D1/S5-P in SCN4A and SCN5A relative to the homologous region in PpSCN1 and D3 domains, appears to correspond to insertion or deletion of N-glycosylation rich sequences bordered by the conserved N-glycosylation site A at the 5' end and the mammalian specific intron at the 3' end. These N-glycosylation rich sequences add 6 and 2 glycosylation sites in SCN4A and SCN5A, respectively (fig.5-6).

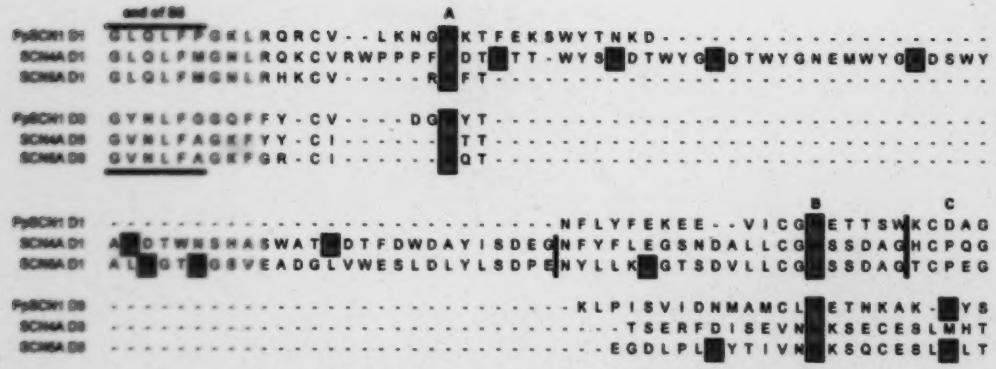

Figure 5-6. Aligned amino acids of the N-glycosylation rich extracellular loops between segment 5 and the membrane-penetrating, pore-forming P segment between segments 5 and 6 in domains 1 and 3 for Na^+ channel genes PpSCN1 (hydrozoan jellyfish), SCN4A (human skeletal muscle) and SCN5A (human heart). Potential N-glycosylation sites are shaded and outlined "N" residues. Conserved N-glycosylation residues between Na^+ channels are labeled A, B and C. A vertical bar is shown where introns interrupt the amino-acid sequences.

Intron/exon boundaries

The majority of the introns of PpSCN1 are present before the first base position of the codon (see Table 5-2), that is phase 0 (65%), with the remainder after the first base position, phase 1 (15%) and after the second base position, phase 2 (20%). The phase positions of exons tend to cluster together, for example, intron placement in phase 2 occurs between exons 2 to 4, and phase 0 between exons 7 to 12 and 14 to 19. This creates an excess of symmetrical exons (63%) exons that begin and end with the same phase.

19 of 20 introns in PpSCN1 conform to the standard U2-type splice junction sequences in eukaryotes, delimited by donor and acceptor site sequences with GT and AG respectively (Table 5-2) (Padgett *et al.*, 1986) with a consensus for the 5' splice donor sequence of GTAAGT, with a match with the consensus at each position, respectively 100%, 100%, 95%, 90%, 70%, 60%, and a consensus for the 3' splice acceptor sequence of TTWYAG with a match to the consensus of 80%, 90%, 85%, 90%, 100%, 100%. 43% of the standard GT-AG introns have potential branch sites that fit the relatively weak consensus for eukaryotes (-YY-TRAY) up to 43 bp upstream from the 3' splice junction, with an average distance of 24 ± 3 bp. With at least 5 of 6 nucleotides matching the branch consensus, there are potential branch points for all the introns between 12 bp to 37 bp upstream from the 3' splice junction with average distance of 22 ± 2 bp.

Intron 2 of PpSCN1, like the homologous intron in most Ca^{2+} and Na^+ channel genes, resembles a class of introns spliced by a rare, U12-type spliceosome (Table 5-3). The homologous intron position in Ca^{2+} and Na^+ channels has either GT-AG or AT-AC sequences delimiting the donor and acceptor sites, with PpSCN1 fitting within the former category (Table 5-3). Intron 2 of PpSCN1 is likely spliced by a U12-type and not the U2-type based on the following characteristics of the intron sequence: 1) There is a perfect match with the U12-type's 5' splice donor sequence (GTATCCTTT). 2) The closest potential branch site (TTTCTTAACACT) closely resembles the more extensive consensus sequence of the U12-type (TTTCTTAACYNY), with matches in 10 of 11 nucleotide positions of the consensus. 3) The branch site consensus lies very close (13 bp) to the 3' splice acceptor site (YAG) like the shorter distances of potential branch sites of the Na^+ and Ca^{2+} channel genes listed in Table 5-3 (range of 9 bp to 12 bp). In comparison, the branch site of the U2-type normally is 18bp to 40 bp upstream from the 3' splice junction in metazoans, but is quite variable and can be found as far as 150 bp upstream (Wu and Krainer, 1996).

Table 3

Consensus Sequences of a Rare U12-type Intron Conserved in PpSCN1 and Most Ca^{2+} and Na^+ Channels[a]

channel type	channel name	5' Donor Splice Site Exon | Intron	Presumptive Branch Site	3' Acceptor Splice Site Intron | Exon
U12-Type AT-AC Introns	consensus:	| [AT][AT][AT]...[AT]..[CT][CT][CT]...[CT][AC] |		
non-L type Ca^{2+}	hCACNL1A4	GAA TGG CC | [AT][AT][AT]...[AT]..[AT][AT][AT]T[AT][AT]----CCAA[CT][AG] | T CCC TTT GAA E W P		P F E
Na^+	hSCN4A	ATC CAT GC | [AT][AT][AT]...[AT]GC..[AT][AT][AT]...[AT][AT]----GCCC[CT][AG] | G CTG TTC AGC I H A		L F S
Na^+	hSCN8A	GTT CAC TC | [AT][AT][AT]...[AT]..[AT][AT][AT]...[AT][AT]-----ACG[CT][AG] | G CTC TTC AAC V H S		L F N
Na^+	mSCN8A	ATA CAT TC | [AT][AT][AT]...[AT]..CC[AT][AT][AT]...[AT][AT]----TCTC[CT][AG] | A GTC TTC AGC I H S		V F S
U12-Type GT-AG Introns:	consensus: T | GTATCCTTT..TTCCTTAACYHY.....YAG | A			
L-type Ca^{2+}	hCACNL1A1	GAA TGG AA | G[AT][AT][AT]...[AT]C..[AT][AT][AT]...[AT][AT]--CTTTCT[CT][AG] | A CCA TTT GAA E W K		P F E
L-type Ca^{2+}	hCACNL1A2	GAA TGG AA | G[AT][AT][AT]...[AT]..[AT]A[AT][AT]...[AT]A[AT]A--TTTTTT[CT][AG] | A CCA TTT GAC E W K		P F E
L-type Ca^{2+}	hCACNL1A3	GAA TGG AA | G[AT][AT][AT]...[AT]C..C[AT][AT][AT]...[AT][AT]----GCTC[CT][AG] | G CCC TTC GAG E W K		P F D
Na^+	PpSCN1	ACT CAT CA | G[AT][AT][AT]...[AT]..[AT]T[AT][AT][AT]...[AT]A[AT]TTACTAAA[CT][AG] | A TAT TTT GAA T H R		Y F E
Na^+	mSCN10A	GTC CAC TC | G[AT]G[AT][AT]...[AT]..[AT][AT][AT]...[AT]ATG-GACCTCA[CT][AG] | C TGG TTC TCC V H S		W F S
U2-Type GT-AG Introns:	consensus: | GTRAGT.....YY.TRAY...YYYYYYY.YAG | G			
L-type Ca^{2+}	**Egl-19	GAA TGG AA | [AT][AT][AT]AGT[AT][AT]ATT	ACCATTTT[CT][AG] | A CCA TTC GAG E W K	P F D
			GT[AT][AT]TTC[AT][AT]A[AT][AT]A AT[AT][AT]TAC[AT][AT]AAT[AT]AAA	

*Shaded residues match the U12-Type AT-AC intron consensus

**The complete intron sequence is shown for the nematode gene (*Egl-19*) with two possibilities for the branch site consensus

DISCUSSION

PpSCN1 and the evolution of Na^+ channel genes

Voltage-dependent Na^+ channels constitute a multi-gene family of channels, likely with members in all metazoan phyla, from cnidarians to mammals (Goldin, 1995). To date, the exon-intron structure has been determined for four out of twelve of the Na^+ channel isoforms that have been characterized in mammals (Goldin, 1995). Three mammalian genes in which the genomic structure is known are associated with genetically-related diseases: hyperkalemic periodic paralysis and paramyotonia congenita (SCN4A) (Cannon, 1994; George *et al.*, 1993; McClatchey *et al.*, 1992), long QT syndrome (SCN5A) (Wang *et al.*, 1996) and motor endplate disease (SCN8A) (Burgess *et al.*, 1995; Plummer *et al.*, 1997). Outside of mammals, partial genomic structures have been reported for fruit fly genes in *D. melanogaster*, *Para* (Loughney *et al.*, 1989) and DSC1 (Salkoff *et al.*, 1987). Cnidarians are the simplest metazoans with discrete nervous systems and express typical, rapid, transient voltage-gated currents carried exclusively by Na^+. As shown in the phylogenetic tree based on amino-acid sequence homology (fig. 5-2), the cnidarian Na^+ channel genes are deeply branched with respect to the other metazoans. This is consistent with fossil evidence and organism phylogeny which place the divergence of the cnidarians early in metazoan history, ~600-1000 million years ago (Morris, 1993).

The close similarity of sequence among Na^+ channels permits the alignment of many of the regions containing introns, thus allowing us to evaluate whether they are homologous sites. 85% of the intron splice-junctions are conserved between jellyfish and mammalian genes in which the genomic structure has been described. It is a striking level of conservation considering the enormous time since cnidarian and mammalian lineages separated. It is likely that the primary arrangement of most exons is retained from the earliest Na^+ channels in the common ancestor of the metazoans.

Ion channels appear to have evolved in a pattern that is similar to other major multi-gene families (Iwabe *et al.*, 1996). Gene duplications and subsequent divergences lead to novel paralogues with distinctive functions, like L, non-L and T-type Ca^{2+} channels and Na^+ channels, before the protostome-deuterostome separation (fig. 5-2) (Ohta, 1991; Ohta, 1994). Paralogues subsequently evolved within sodium and calcium channels, giving rise to further branching, such as the A, B, E, C, D and S classes in calcium channels. In Ca^{2+} channel genes of L-type, (class C, D, S) and non-L type Ca^{2+} channels in mammals (class A), one quarter of the intron sites are in homologous positions to those in PpSCN1 and mammalian Na^+ channel genes. This indicates that some of the introns are probably descended from a common ancestor of the L and non-L type Ca^{2+} channels and Na^+ channels. The original form of the first Ca^{2+} channel is unclear, but since there is a single cation channel gene in yeast genomes with a shared Na^+ and Ca^{2+} channel homology (Van Der Aart *et al.*, 1996), it is possible that the four-

domain ancestor originated before the radiation of eukaryotes (protists, plants, fungi, animals), at least a billion years ago.

Structural evolution within the sodium channel gene family

Comparative analysis among channels provides an approach to evaluating the tolerance of structural features to evolutionary changes, and to hypothesizing which differences are due to adaptive structural changes taken by different channel types. In general the level of similarity between Na^+ channel regions can be ranked into three groups: 1) Highly conserved regions, specifically the membrane spanning portions of the domains (D1 through D4), and the short, cytoplasmic linker ID3-4; 2) Regions of intermediate similarity found in the extracellular loops; 3) Regions of lowest similarity in the long intracellular loops ID1-2 and ID2-3, and the amino- and carboxyl- termini (Catterall, 1996; Fozzard and Hanck, 1996).

Ca^{2+} channels (L and non-L types) share a similar pattern of sequence conservation as Na^+ channels, but differ by having approximately twice as many introns as Na^+ channels (Hogan *et al.*, 1994; Ophoff *et al.*, 1996; Peixoto *et al.*, 1997; Soldatov, 1994; Yamada *et al.*, 1995). Over one-third of these introns are found in the relatively long C-terminus of most Ca^{2+} channels, after the position of the last intron in Na^+ channels (DIV, S3). In all cases the intron sites conserved among Ca^{2+} and Na^+ channels are found in the most highly conserved regions of the protein sequence while the intron

sites that are unique to specific genes are found in the unconserved regions. The uniqueness is not due to an inability to align highly divergent sequences, since the number and phase of those introns in the long extracellular loop, D1/S5-P and intracellular loops ID1-2 and ID2-3 differ among the various channels.

The low sequence similarity suggests that these regions are not involved in the fundamental and shared characteristics of Na^+ and Ca^{2+} channels such as ion selectivity, voltage-sensing, activation and inactivation (Catterall, 1996). The structural variety of these regions probably represents a range of changes during evolution, some that led to features tailored to individual cellular environments.

For example, the highly divergent ID1-2 has a range of sizes (see fig. 5-5) from PpSCN1 (97 aa) to the long cytoplasmic loop in the fruit fly gene, *Para* (384 aa). Phosphorylation of the long ID1-2 loops found in the Na^+ channels of rat brain SCN2A (329 aa) and heart SCN5A (299 aa) at protein kinase A (PKA) sites results is an increase or decrease in the size of the channel current amplitude (Cukierman, 1996). In rat heart cells, activation of PKA, causes Na^+ channels to become more Ca^{2+} permeable (Santana *et al.*, 1998). *Para* contains two consensus PKA sites, one of which is contained in an alternatively spliced exon A, shown to be associated with expression of high Na^+ current density (O'Dowd *et al.*, 1995). On the other hand, SCN4A has a short ID1-2 loop (126 aa) similar in size to PpSCN1 and contains no consensus sites for phosphorylation by PKA. The high variability in size, sequence and number of PKA sites in ID1-2, suggest

that phosphorylation site-rich fragments in ID1-2 have evolved in each channel subtype for specialized regulatory roles.

Like ID1-2, the extracellular loop S5-P in D1 is variable among Na^+ channels, ranging from 72 aa (PpSCN1) to 121 aa (SCN4A). S5-P in Na^+ channels is also approximately twice the size in D1 (mean ± S.E.M. = 86 ± 2.69, for n=20[12]) than the corresponding region in D3 (42 ± 0.85 for n=20[10]). Despite their differences in size and sequence in Na^+ channels, an obvious kinship between the extracellular loops in D1 and D3 is evident by the high density of N-linked glycosylation sites in both these loops (average ± S.E.M. = 6.2 ± 1.2 sites, range = 4 to 10 sites, n=20[10]) and the presence of two conserved glycosylation sites in homologous positions in Na^+ channels from jellyfish to mammals. In an alignment of D1/S5-P and D3/S5-P, it appears that N-glycosylation rich sequences between the conserved N-glycosylation sites in the mammalian Na^+ channels are largely responsible for the major differences in extracellular loop sizes in D1 between these channels and PpSCN1 (fig. 5-6). The role of N-glycosylation in Na^+ channels is not clear, but in general N-glycosylation is reported to stabilize the proteins' secondary structures, folding, processing and directing newly-manufactured channels to the plasma membrane (Fozzard and Hanck, 1996). Differences between the density and distribution of N-glycosylation sites may be key elements in differential expression of channel subtypes. Furthermore, the closeness of the N-linked glycosylation sites to the pore-forming P-loop, in D1 and D3, suggests that channel differences in sialation may be

[12] Na^+ channel genes are the same as those in the phylogenetic tree (fig. 5-2).

responsible for differences in ion permeability and the effectiveness of pore-occluding toxins (Recio-Pinto *et al.*, 1990).

Thus it appears that changes in the numbers and locations of introns are associated with the large evolutionary changes seen in regions of low sequence conservation.

Genetic diversity and alternative-splicing in sodium channels

The complete genomic region spanning the Na^+ channel cDNA of PpSCN1 has been sequenced. This gene does not appear to contain alternative-spliced variants. Northern and Southern blot analysis support this. Only a single ~6.0 kb mRNA transcript is discernible on a Northern blot, using cDNA and genomic probes created from the 5' and 3' ends of the Na^+ channel, respectively (see Chapter 4), and only the predicted PpSCN1 gene is evident using 5 genomic probes based on fragments along most of the Na^+ channel (fig. 5-1). Synonymous substitutions were found in the DNA sequence in PpSCN1 and are likely polymorphisms since tissues were pooled from many jellyfish to construct the cDNA and genomic libraries and most of the differences that appear between the cDNAs (9 out of 15) were identified in the two genomic clones, with PpSCN1.1g corresponding to cDNA fragments PpSCN1.1Ac and PpSCN1.1Bc, and PpSCN1.2g to cDNA fragments PpSCN1.2Ac and PpSCN1.2Bc. Numerous sequence variations have been reported for other cation channel genes and were considered either

polymorphisms, cloning artifacts or possible variations due to post-transcriptional RNA editing (George *et al.*, 1993; Ophoff *et al.*, 1996; Peixoto *et al.*, 1997).

Alternatively-spliced variants of Na^+ channels have been found in a few vertebrate genes (SCN2A, SCN3A (Belcher *et al.*, 1995; Gustafson *et al.*, 1993; Sarao *et al.*, 1991; Schaller *et al.*, 1992) and SCN8A) (Burgess *et al.*, 1995), but so far, there is considerably more diversity due to the presence of at least a dozen separate pore-forming α subunit genes (Goldin, 1995). In comparison, we have been able to isolate only the single gene PpSCN1 in *P. penicillatus* (see Chapter 4) and no more than two pore-forming α subunit genes have been detected so far from any single invertebrate species (cnidarians, nematodes, arthropods, mollusks). The fruit fly, for example, appears to use post-transcriptional modification of the same gene as a strategy to generate genetic diversity in Na^+ channels (see *Para*, fig. 5-5) as well as in *Shaker* K^+ channels (Kamb *et al.*, 1988; Schwarz *et al.*, 1988) and Ca^{2+} channels (Peixoto *et al.*, 1997). Without alternative-splicing in Na^+ channels, it may be that *P. penicillatus* compensates for the lack of genetic diversity in α subunits by alterations in phosphorylation (Cukierman, 1996) and or activity of accessory subunits as has been shown for other Na^+ channels (Adelman, 1995).

The question of whether alternative exons originate as far back as the two domain precursor of Ca^{2+} and Na^+ channels has been raised by Plummer and coworkers (1997). They discovered that similarly placed protein segments, which span part of segment 3 and segment 4 in D1 and D3 of the vertebrate Na^+ channel SCN8A, are each encoded by

alternative (neonatal and adult) exons, with a relatively short intron separating the neonatal form, which lies upstream from the adult form in the gene. The positions of the alternative exons in SCN8A are equivalent to exons numbered 5 and 16 in PpSCN1. If exons numbered 5 and 16 in PpSCN1 are to be regarded as homologues, intron-splice junctions must have "slipped" nucleotide positions 5 bp and 8 bp, respectively from each other since D1 and D3 diverged in the two domain precursor of Ca^{2+} and Na^+ channels (fig. 5-7). If the shared homology of exon 5 and 16 in PpSCN1 extends to Ca^{2+} channels, then the equivalent splice site numbered 15 in PpSCN1 for Ca^{2+} channels would either have "slipped" at least 26 bp, or have been secondarily lost in Ca^{2+} channels (fig. 5-7). Apparent intron slippage is not likely due to an alignment ambiguity of amino-acid sequences because there is also a difference in intron phase for each pair.

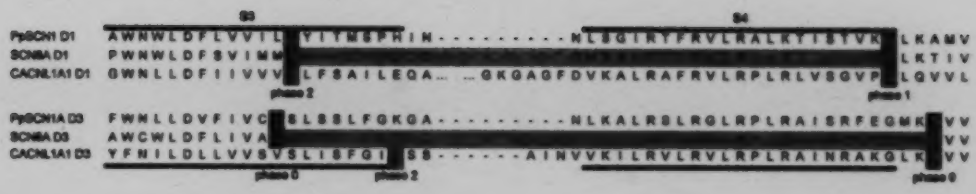

Figure 5-7. Positions of introns in an alignment of amino acid sequences from segments 3 and 4 in domains 1 and 3, with the position (inverted residues) and phase of introns indicated for Na^+ channel genes PpSCN1 (hydrozoan jellyfish) and SCN8A (mouse brain and spinal cord), and an L-type Ca^{2+} channel gene (CACNL1A1). The alternatively transcribed exons in adult mice in domain 1 (5A) and domain 3 (18A) of SCN8A are shaded. To save space, ten amino acids are removed in CACNL1A1 between S3 and S4 in D1.

Allowances for intron slippage (up to 12 bp) has been advocated by those proposing an early appearance of introns in evolution, because it provides a greater percentage of introns in homologous positions between prokaryotes, Archaebacteria and eukaryotes (Gilbert and Glynias, 1993; Long *et al.*, 1995). Recently, allowances for intron slippage and the "introns early" viewpoint have been criticized because of the extremely high slippage rates of introns that is required to correlate boundary positions of protein coding units, and a lack of evidence that intron slippage is a wide-spread phenomenon (Logsdon *et al.*, 1995; Rzhetsky *et al.*, 1997; Stoltzfus *et al.*, 1997). Without intron slippage it is difficult to argue for the possible homology of alternative exons (Plummer *et al.*, 1997), or introns (George *et al.*, 1993) in the two domain precursor of Ca^{2+} and Na^+ channels. More definitive support for an early appearance of alternative exons or introns in a two domain precursor of Ca^{2+} and Na^+ channels may come from a more extensive phylogenetic survey of genomic structures in Ca^{2+} and Na^+ channels. At the present time, outside of the closely-related SCN2A, SCN3A and SCN8A Na^+ channels in mammals, only a *Drosophila* non-L type Ca^{2+} channel contains a homologously-placed, alternative-spliced variant for exon #5 in PpSCN1 (Peixoto *et al.*, 1997) and only vertebrate orthologues of SCN8A contain multiple alternative-spliced variants of exon #16 in PpSCN1 (Plummer *et al.*, 1997).

Evolution of the U2 and U12 spliceosome mechanisms in calcium and sodium channels

The homologous intron in SCN4A to intron #2 of PpSCN1 has been shown to be processed by a rare "U12-type" pre-mRNA splicing machinery (Hall and Padgett, 1996; Tarn and Steitz, 1996; Wu and Krainer, 1996). This type of intron uses a different set of small nuclear ribonucleoproteins to the standard U2-type and is more rare, likely represented by less that 0.1% of total introns (Sharp and Burge, 1997).

Based on intron sequences, the U12-type spliceosome mechanism appears to be retained in the homologous position in almost all members of the voltage-gated Ca^{2+} channels (L and non-L type) and Na^+ channels, from jellyfish to mammals. The stability of this splice site is striking when it is considered that it is retained throughout the evolution of two functionally different metazoan ion channel classes at a single site. Having two mechanisms in a single gene means that shuffling during non-homologous DNA recombination will juxtapose intron-exon boundaries that are spliced under different, incompatible spliceosome mechanisms (Sharp and Burge, 1997). The incompatibility of the two splicing mechanisms has been demonstrated for the homologous U12-type intron in the mouse Na^+ channel gene, SCN8A, where mutations at the mouse locus "motor endplate disease", lead to a possible matchup of U12 and U2-type splice sites resulted in exons being skipped in order to maintain compatible donor and acceptor splice sites (Kohrman *et al.*, 1996).

It has been proposed that the U12-type is the rare splicing mechanism because of its more extensive recognition sequence at the 5' donor splice and branch sites compared to the U2-type (Kohrman *et al.*, 1996). The accumulation of mutations in consensus sequences should, over time, lead to a conversion of a U12-type splice site to one recognized by a U2-type rather than to the modifications required to convert a U2 to a U12-type (Sharp and Burge, 1997). We surveyed genomic sequences for this family of ion channels and found one apparent case of the conversion of the U12 to U2-type in a *C. elegans* clone described by (Lee *et al.*, 1997). *Egl-19* appears to operate under the U2-type splicing mechanism, based on the recognition sequences that are closer to the consensus for the U2-type than the U12-type splicing mechanism (Table 5-3). The 4^{th}, 5^{th} and 6^{th} position from the 5' donor sequence of the U12-type are invariant as TCC in all known genes (Sharp and Burge, 1997), but *Egl-19* has AGT which matches the U2-type consensus. The whole intron is displayed for *Egl-19* in Table 5-3, but the two possibilities for the U12-Type branch site are a poor fit.

Voltage-gated Ca^{2+} and Na^+ channels have a wide distribution of AT-AC and GT-AG sequences delimiting the donor and acceptor sites in the U12-type intron of voltage-gated Na^+ and Ca^{2+} channels, suggesting that the conversion between GT-AG and AT-AC appears to be common. GenBank database searches reveal that AT-AC and GT-AG are present in introns for both the U2 and U12-type introns but the AT-AC boundaries are much less abundant (Dietrich *et al.*, 1997; Sharp and Burge, 1997). One rare U2-type intron, with AT-AC boundaries, was identified in introns numbered 21 and 25 in Na^+

channels SCN4A and SCN5A, respectively, located at the 5' end of the conserved, cytoplasmic linker between domains III and IV (Wu and Krainer, 1997). This rare AT-AC, U2-type intron may have been introduced in Na^+ channels and secondarily lost in PpSCN1, since this intron appears to be missing in Ca^{2+} channels but present in at least one Na^+ channel of fruit fly (DSC1) and in the sea hare, *Aplysia california* (Dietrich *et al.*, 1997).

A comparative approach provides valuable insights into the understanding of the structure and function of voltage-gated Na^+ channels. In this chapter it is shown that the arrangement of exons and different intron splicing mechanisms are remarkably conserved from jellyfish to mammalian Na^+ channels, with partial conservation of intron positions in L and non-L type Ca^{2+} channels. Regional differences in Na^+ channels appear to correspond to potential adaptive roles among channel types, for example, phosphorylation by protein kinase A, glycosylation and post-transcriptional modification. Outside of the specialized modifications in Na^+ channel genes, the organization and fundamental composition of the genetic template for Na^+ channels has undergone few changes since it was laid down in the common ancestors of diploblasts and triploblasts, ~600 million to 1 billion years ago (Morris, 1993).

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CHAPTER 6 GENERAL DISCUSSION AND CONCLUSIONS

IMPLICATIONS FOR THE EVOLUTION OF VOLTAGE-GATED SODIUM CHANNELS FROM A STUDY OF SODIUM CURRENTS AND CHANNELS FROM THE HYDROZOAN JELLYFISH, *POLYORCHIS PENICILLATUS*

This thesis describes a study of cnidarian Na^+ channels and currents using molecular biological and electrophysiological techniques, undertaken with the aim of looking for clues concerning the evolution of Na^+ channels. It features a detailed description of a sodium current in swimming motor neurons (SMN) (Chapter 2 and 3), a partial description of another sodium current in B neurons (Chapter 3), and the structure of a full-length sodium channel gene and transcript (Chapter 4 and 5) from *Polyorchis penicillatus*.

Functional comparisons between currents from jellyfish and triploblasts, and alignments of their genes underscore possible structural-functional similarities and differences. Similarity of features of the jellyfish gene and currents to those in triploblasts is suggestive of possible ancestral features that may be present in Na^+ channels of the first nervous system-bearing animals, the common ancestor of cnidarians and other metazoans that lived ~600 million to 1 billion years ago (Morris, 1993). The use of cnidarians as a means to provide insight into evolution of metazoan nervous

systems is best stated by George Mackie, "*There really is no better group in which to look for clues*"[13].

The four most important issues that were addressed in the first five chapters of this thesis will be discussed in this, the final and sixth chapter. These four issues are phrased as questions (A to D):

A) What are the common features of sodium channels in jellyfish and other metazoans? Did Na^+ channels of jellyfish and mammals arise from the same gene in a common ancestor?

B) What are the features of Na^+ channels that are shared with Ca^{2+} channels, the presumed ancestors of Na^+ channels? Do Na^+ channels in jellyfish resemble Ca^{2+} channels more than other Na^+ channels?

C) How does the structural and functional diversity of Na^+ channels in *P. penicillatus* compare to the diversity in mammals? Why are there functionally diverse Na^+ currents in jellyfish? What are the structures that are responsible for the electrophysiologically separable Na^+ currents described in SMNs and B neurons (Chapter 3)?

D) What are the major differences between jellyfish and other metazoan Na^+ channels? Are the poorly conserved regions the result of changes arising from the

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[13]Excerpt from the first paragraph addressed at the plenary lecture by the "grandfather of jellyfish neurobiology" George O. Mackie for the NATO Advanced Research Workshop: "Evolution of the First Nervous Systems", St. Andrews University, Scotland, July 1989. (See title page for full quotation).

absence of selection acting upon channel types or are some of the differences between different channels due to unique adaptations of individual channel types?

The last section, E, of this final chapter incorporates the research findings in this thesis in a unifying proposal for the evolution of voltage-gated ion channels.

A. Common features between jellyfish and mammalian Na^+ channels

Na^+ ions play a role as the major inward current responsible for the upstroke of action potentials in swimming motor neurons (SMNs) of the jellyfish *Polyorchis penicillatus* as in most neurons of metazoans. In Chapter 3, I show that the plateau of SMN spikes is maintained mostly by sodium influx since the very slowly inactivating component of the Na^+ current in SMNs is almost two orders of magnitude larger than the sustained Ca^{2+} current, HVA-s. Maintenance of the plateau by Na^+ ions has functional consequences since the variably expressed plateau phase has been shown to modulate transmitter release onto the post-synaptic, epithelio-muscular cells (Spencer *et al.*, 1989; Spencer, 1982). Considering that the cnidarians are the simplest extant organisms to have Na^+-dependent action potentials and a discrete nervous system, it is likely that the first appearance of Na^+ currents as major carriers of action potentials occurred in nervous systems of ancient animals resembling the cnidarians. The next question to be addressed

is whether cnidarian and Na^+ channels of other metazoans are derived from the same gene in common ancestors.

The structural features of Na^+ channels contained in the major membrane-spanning domains and short cytoplasmic linker between domains III and IV, where there is a high degree of similarity (~70%) between the putative amino acid sequence of the jellyfish clone PpSCN1 and representative Na^+ channels from non-cnidarian species (see Chapter 4), are implicated in the fundamental properties of Na^+ channels: ion selectivity, voltage-sensing, activation and inactivation.

The critical residue differences that are considered to distinguish the pores of Na^+ channels from Ca^{2+} channels in mammals (Heinemann *et al.*, 1992) are similar but not identical to the residue differences in the jellyfish Na^+ channel PpSCN1 (Chapter 4). A highly conserved pore was also suggested by the very similar relative permeabilities to monovalent cations in SMNs when compared with Na^+ channels in other species (Chapter 3).

Besides ion selectivity, other major features that appear to be conserved in jellyfish Na^+ channels include the property of channel opening (activation) and subsequent inactivation. In Na^+ currents of SMNs, the rate of activation was typical of other metazoan Na^+ currents in being rapid and steeply voltage-dependent (see Chapter 3). The structural elements that current models propose to be responsible for voltage-sensitive opening of Na^+ channels are conserved in the jellyfish Na^+ channel PpSCN1

with repeating positive charges every third amino acid in the 4th transmembrane segments of each of the four domains (Chapter 4).

Inactivation, as for many other Na^+ currents, was rapid and could be fitted with two exponents in SMNs (Chapter 4). The fast component of inactivation is thought to involve the short highly conserved cytoplasmic loop between domains III-IV, which is 54 amino acids long in the jellyfish Na^+ channel clone PpSCN1 and is the same size (53 or 54 amino acids) in most Na^+ channels (Catterall, 1996). In particular, the most critical residues of the inactivation receptor for fast inactivation decay are three hydrophobic amino acids (IFM) that are conserved in almost all known Na^+ channels (Catterall, 1996), including the jellyfish channel, PpSCN1.

Not only is there similarity in the structure and function of jellyfish and mammalian Na^+ channels, but the organization of exons (coding regions) in Na^+ channel genes are also very similar (Chapter 5). 85% of the intron splice sites partition the coding region in the same homologous position in mammalian channels from human skeletal muscle SCN4A, human heart SCN5A and mouse sensory neurons SCN10A (Chapter 5). The intron positions are highly conserved in Na^+ channels from jellyfish to mammals, in spite of the lack of similarity of intron sequences and large differences in intron sizes among the Na^+ channel genes (Chapter 5).

One intron in the jellyfish Na^+ channel PpSCN1, intron 2, is homologous to an intron in a Na^+ channel gene from human skeletal muscle (SCN4A) that is known to be spliced by an extremely rare U12 type spliceosome likely represented by less than 0.1%

of total introns (Sharp and Burge, 1997). Despite the rarity of the intron type, the U12-type splice site appears to be retained in the homologous position in almost all Ca^{2+} and Na^{+} channels, including intron 2 in PpSCN1, based on intron sequences.

The similar arrangement of exons and their different spliceosome mechanisms, the high conservation of major structural features between jellyfish and mammalian Na^{+} channel genes and their recorded currents, suggest that Na^{+} channels likely originated in the common ancestors of diploblasts and triploblasts, ~600 million to 1 billion years ago (Morris, 1993).

B. Conserved features between jellyfish and mammalian Na^{+} channels and Ca^{2+} channels

The immediate ancestral molecules to the Na^{+} channels were likely Ca^{2+} channels with four domains. The kinship of Na^{+} channels and Ca^{2+} channels can be traced in their domain similarities. Each domain of the Na^{+} channel has a greater structural similarity to the corresponding domain in Ca^{2+} channels than to any other of its own domains or to any other gene (Strong *et al.*, 1993).

A common origin of Na^{+} channels and Ca^{2+} channels is also apparent in the placement of intron splice sites. 25% of the intron splice sites found in the jellyfish Na^{+}

channel clone PpSCN1 and mammalian Na^+ channel genes are also found in homologous positions in mammalian Ca^{2+} channels (Chapter 5).

The most parsimonious phylogenetic tree based on aligned amino acid sequences of channel proteins suggests that Na^+ channels resemble T-Type Ca^{2+} channels more than non-L or L-type Ca^{2+} channels (Chapter 5). Na^+ channels also resemble T-Type Ca^{2+} channels kinetically. They are both transient, inward currents that are rapidly activating and inactivating.

As few as two residue differences (Heinemann *et al.*, 1993) or phosphorylation of residues (Santana *et al.*, 1998) in the pore separate Ca^{2+} from Na^+ selectivity. With such small differences distinguishing Na^+ and Ca^{2+} channels, in comparison to the large sizes of each of the proteins, one may expect channels to exist with characteristics of both channel types, including a channel with intermediate selectivity or pharmacology between Na^+ and Ca^{2+} channels. A suitable place to start looking for hybrid Ca^{2+}-Na^+ channels is in the cnidarians, given their basal position in the Metazoa and the apparent simplicity of their nervous system.

In SMNs, Na^+ currents are not sensitive to typical Na^+ channel drugs, including tetrodotoxin (Chapter 2). However, despite their lack of definition by conventional Na^+ channel pharmacology, they do not have a pharmacological profile that is similar to Ca^{2+} channels either. In SMNs, Na^+ currents are clearly distinct from Ca^{2+} currents in their kinetics and pharmacology (Chapter 2), as are most Na^+ and Ca^{2+} currents observed in neurons of other metazoans (Hille, 1992).

The structure of the Na^+ channel gene, PpSCN1, from *P. penicillatus* also is clearly distinct from Ca^{2+} channels. PpSCN1 is deeply branched in the most parsimonious phylogenetic tree of Na^+ and Ca^{2+} channels, but groups with other Na^+ channels in overall structural similarity. PpSCN1, like all Na^+ channels isolated to date, is more distantly related to Ca^{2+} channels than it is to other Na^+ channels (Chapter 4 and 5).

Consistent with the clear placement of jellyfish Na^+ channels in the classification of metazoan Na^+ channels is the placement of the other voltage-gated ion channels studied in *P. penicillatus*. Subfamily members of voltage-gated K^+ channels such as the *Shaker* and *Shal* relatives have been cloned from *P. penicillatus* and expressed in *Xenopus* oocytes (Jegla *et al.*, 1995; Jegla and Salkoff, 1997). The characteristics of the expressed K^+ currents resemble the fly and mammalian homologues to such a degree that they have been used as alternative models for studying voltage-sensitivity (Grigoriev *et al.*, 1997), K^+-sensitive C-type inactivation (Grigoriev *et al.*, 1998) and modulation of K^+ channels by accessory subunits (Jegla and Salkoff, 1997). Ca^{2+} channel homologues are also likely to be present in *P. penicillatus*, given that an L-type Ca^{2+} channel has been recently cloned and expressed in *Xenopus* oocytes from a scyphozoan jellyifish, *Cyanea capillata* (Jeziorski *et al.*, 1997a). Thus, all things considered, it appears that the diversification of the ion channel families (K^+, Ca^{2+} and Na^+) precedes the appearance of cnidarian-like forms at a time before or during the earliest period of the metazoan radiation. This in turn suggests that a transitional Na^+-Ca^{2+} channel hybrid is probably

not present in Cnidaria because they have a "fully evolved" suite of differentiated ion channels.

C. Diversity of sodium channels and currents in *Polyorchis penicillatus*

Sodium channels do not appear to have as much structural and functional diversity as the other voltage-gated ion channels, the K^+ and Ca^{2+} channels. The relative absence of diversity in Na^+ channels likely relates to its very specialized role in high frequency signaling and rapid propagation (Hille, 1992). Nonetheless, sodium channels do show diversity in functional and pharmacological properties. Many voltage-gated sodium currents have been differentiated by their inactivation kinetics, ranging from very fast (Hille, 1992), to slow (Alonso and Llinas, 1989), to non-inactivating (Taylor, 1993). Different inactivation kinetics of sodium channels can influence action potential shape and play significant physiological roles in mammals (Campbell, 1992).

In *P. penicillatus*, at least three electrophysiologically distinguishable sodium currents are present. There is a slowly activating and slowly inactivating sodium current in the electically excitable epithelium, which is inhibited by high concentrations of internal calcium (Grigoriev and Spencer, 1996). In contrast, "B" neurons in *P. penicillatus* have short duration, sodium-dependent action potentials (Spencer and Arkett, 1984). In Chapter 3, it was shown that the sodium current responsible for the action potential of "B" neurons shows rapid inactivation and recovery from inactivation (<5 ms)

(Grigoriev *et al.*, 1996). The rapid inactivation recovery rate enables "B" neurons to fire at frequencies as high as 10 Hz (Spencer and Arkett, 1984). Lastly, in Chapter 3 that are kinetically similar to mammalian cardiac channels, with rates of recovery from inactivation ~10 fold slower than typical mammalian neurons (Grigoriev *et al.*, 1996), were described in swim motor neurons (SMNs). The non-neuronal like morphology of SMNs and the slow inactivation recovery rate of their Na^+ currents indicate that these neurons have properties that are more "cardiac-like" than "neuron-like". An argument can be made that the non-neuronal properties of the "cardiac-like" current in SMNs may have arisen by convergence since in both cases, the spread of excitatory waves occurs in a syncytial network that is prone to re-entry. In the heart and network of SMNs, a slow recovery rate from inactivation prevents re-entry and disruption of the rhythmic input that leads to synchronous contractions of the heart and jellyfish bell (Fye, 1987).

In mammals, expression of different α subunit genes is responsible for the electrical and pharmacological differences between neuronal, skeletal muscle and cardiac sodium channels (Goldin, 1995). A least a dozen mammalian sodium channel genes are known, many of which have alternatively-spliced variants (Goldin, 1995). The properties of sodium channels in mammals are also modified by the presence of accessory subunits and by phosphorylation (Adelman, 1995), thus creating ample structural and hence functional diversity.

In comparison, no more than two α subunit genes have been detected so far from any single invertebrate species (cnidarians, nematodes, mollusks, insects). Even though

jellyfish have functionally diverse sodium currents, PpSCN1 is the only sodium channel α subunit in *P. penicillatus* that we have detected (Chapter 4 and 5).

In mammals the kinetic properties of α subunits can be modified by co-expression with small accessory, ß subunits (Goldin, 1995). Functional expression of the *Drosophila* α subunit requires an accessory subunit, TipE that is not homologous to the mammalian ß subunit (Feng *et al.*, 1995). We have not yet attempted to express PpSCN1, but it is probable that, like the other invertebrate α subunit clones (cnidarian (Anderson *et al.*, 1993; White *et al.*, 1998), flatworm (Jeziorski *et al.*, 1997b) and molluscan (Dyer *et al.*, 1997; Rosenthal and Gilly, 1993; Sato and Matsumoto, 1992)), it requires unique accessory subunits for functional expression.

Although no alternatively spliced variants were found in the jellyfish gene, PpSCN1 (Chapter 5), one invertebrate, the fruit fly, *Drosophila melanogaster*, appears to use alternative splicing of a single gene as a strategy to generate functional diversity in Na^+ channels (see *Para*, fig. 5-5) as well as in *Shaker* K^+ channels (Kamb *et al.*, 1988; Schwarz *et al.*, 1988) and Ca^{2+} channels (Peixoto *et al.*, 1997). Without alternative-splicing in Na^+ channels, it may be that *P. penicillatus* compensates for the lack of genetic diversity in α subunits by alterations in phosphorylation (Cukierman, 1996) and or activity of accessory subunits as has been shown for other Na^+ channels (Adelman, 1995). It also may be that there are other sodium-selective channel genes present in *P. penicillatus* with sufficiently different structures that the molecular techniques used to

isolate PpSCN1, which were designed to clone a sodium channel gene of close similarity to known channels, could not detect.

D. Adaptive features of jellyfish and other metazoan Na^+ channels

Comparative analysis among channels provides an approach to evaluate the tolerance of structural features to evolutionary changes, and to hypothesize which differences are due to adaptive structural changes taken by different channel types. Between Na^+ channels, major differences in sizes and sequences exist in the amino- and carboxyl-termini, the extracellular loop in domain I (D/S5-P), and the cytoplasmic loops between domains I and II (ID1-2) and between domains II and III (ID2-3) (Chapter 4 and 5).

In all cases, the intron sites conserved among jellyfish and mammalian Na^+ channels are found in the most highly conserved regions of the protein sequences while the intron sites that are unique to specific genes are found in the unconserved regions (Chapter 5). The differences between Na^+ channels are not solely due to an inability to align highly divergent sequences, since the number and phase of those introns in the long extracellular loop, D1/S5-P and intracellular loops ID1-2 and ID2-3 differ among the various channels.

The differences between the jellyfish and mammalian genes correspond to sequences that are rich in phosphorylation sites and structural motifs for binding cytoplasmic proteins (cytoplasmic loops ID1-2, ID2-3 and the carboxyl-terminus) and glycosylation sites (extracellular loop in domain I) (Chapter 5).

Phosphorylation of the long cytoplasmic loops in ID1-2 in mammalian brain and heart channels at protein kinase A (PKA) sites leads to a change in the current size of the channels (Cukierman, 1996). In mammalian heart cells, activation of PKA causes Na^+ channels to become more Ca^{2+} permeable (Santana *et al.*, 1998). *Para*, a Na^+ channel from fruit fly contains two consensus PKA sites, one of which is contained in alternatively spliced exon A, shown to be associated with expression of high Na^+ current density (O'Dowd *et al.*, 1995). On the other hand, the Na^+ channel in mammalian skeletal muscle has a short cytoplasmic loop like the jellyfish channel PpSCN1 and contain no consensus sites for phosphorylation by PKA. The high variability in size, sequence and number of PKA sites, suggest that phosphorylation site-rich fragments in ID1-2 have evolved in each channel subtype for specialized regulatory roles.

Like ID1-2, the extracellular loop in D1 (D1/S5-P) is variable in size, yet has an obvious kinship with the corresponding extracellular loop in D3 by the high density of N-linked glycosylation sites in both of these loops and the presence of two conserved glycosylation sites in homologous positions in Na^+ channels from jellyfish to mammals. In an alignment of the extracellular loops in domains I and III (D1/S5-P and D3/S5-P), it appears that N-glycosylation rich sequences between the conserved N-glycosylation sites

in the mammalian Na^+ channels are largely responsible for the major differences in extracellular loop sizes in domain I between these channels and PpSCN1 (Chapter 5). The role of N-glycosylation in Na^+ channels is not clear, but in general N-glycosylation is reported to stabilize the proteins' secondary structures, folding, processing and directing newly-manufactured channels to the plasma membrane (Fozzard and Hanck, 1996). Differences between the density and distribution of N-glycosylation sites may be key elements in differential expression of channel subtypes. Furthermore, the closeness of the N-linked glycosylation sites to the pore-forming P-loops in domains I and III, suggest that channel differences in sialation may be responsible for differences in ion permeability and the effectiveness of pore-occluding toxins (Recio-Pinto *et al.*, 1990).

Thus it appears that changes in the numbers and locations of introns are associated with large evolutionary changes seen in regions of low sequence conservation. These changes appear to correspond to specialized features of individual channel types.

E. HYPOTHETICAL EVOLUTION OF VOLTAGE-GATED ION CHANNELS

The time will come I believe, though I shall not live to see it, when we shall have very fairly true genealogical trees of each great kingdom of nature.

Charles Darwin

The advent of molecular cloning has produced large databases of cloned genes across the major kingdoms of life, making it attractive to build phylogenetic relationships of ion channels from trees generated from gene structures and functional characteristics gathered from electrophysiology.

A. Structural evolution of ion channels

The simplest K^+ channels have two transmembrane segments (2TM) bounding the pore-forming, P-loop and belong to the inward rectifier family in metazoans (fig. 6-1). They also can be thought of as the most functionally simple, stripped down channels consisting primarily of the K^+-selective elements, which in the absence of an extracellular block by divalent cations, are open at all potentials (Hille, 1992). The other structural classes appear as modular additions to this two-transmembrane (2TM) construction (fig.

6-1). Probable derivatives of the 2TM structural class are the 4TM, double-barreled pored K^+ channels (fig. 6-1).

The 6TM class of K^+ channels are voltage-sensitive, which is conferred primarily by extra segments (S1 through S4) to the 2TM construct. The signature feature of voltage-sensors are positively-charged amino acids every third residue in 4th segments typified by the *Shaker* subclass and Ca^{2+} and Na^+ channels (Table 6-1).

Each class of the ion channels can be differentiated by their trademark ion selective pore. Mapping by open channel blockers, cysteine-scanning mutagenesis (Sather *et al.*, 1994) and most recently analysis by X-ray crystallography (Doyle *et al.*, 1998) have established that the pore of these channels has a very narrow selectivity filter formed by residues in the P-loop (see highlighted residues in Table 6-2). Site-specific mutations of a very few residues in the selective filter of the P-loop can alter the apparent selectivity of channels from K^+ to more Ca^{2+}-like (Heginbotham *et al.*, 1992), Ca^{2+} to more Na^+-like and Na^+ to Ca^{2+} (Heinemann *et al.*, 1992). These mutagenesis studies of ion channel pores underscore how potentially few residues separate these highly divergent, structural and functional classes. They also hint at an evolutionary progression of the ion selective filter, and the ion channels themselves, from channels that are selective for K^+ to Ca^{2+} to Na^+.

K^+-selectivity appears to be conferred primarily by a "GYG" motif in the P-loop (Doyle *et al.*, 1998). Ca^{2+} channels appear to have gaps in the homologous position of the "YG" of K^+ channels (Table 6-2). They have a pore that resembles the binding sites

of calcium chelators like (ethylenediaminetetraacetic acid) EDTA, with the carboxylic acid groups from the four negatively charged residues, glutamates (E) or aspartates (D) of each domain, forming a high-affinity pocket for Ca^{2+} ions (McCleskey, 1994).

Bacterial species contain both the 2TM and 6TM structural classes, so either class may be more ancient. Possible caveats to an early origin of K^+ channels are that the bacterial channels may be the result of convergence or alternatively, are recent imports from eukaryotes. Both processes are considered rare, but cannot be dismissed. However, it would seem quite improbable for convergence to take place in 6TM K^+ channels of two different bacterial species (Table 6-2) and for an additional 2TM K^+ channel to evolve with the surprisingly conserved K^+ pore-loop with the trademark "VGYGD" motif (Table 6-2). General structural trends are found between the major transport families and ion channels, suggestive of convergence in overall design, but have overall low sequence identities (Jan and Jan 1994; Saier, 1994).

Additional support for an early appearance of 6TM K^+ channels is the phylogenetic support in basal eukaryotes. 6TM K^+ channels are firmly established in the protists with 6 unique genes so far isolated from the *Paramecium tetaurelia*, all of which have the trademark "VGYGD" selective pore (Jegla and Salkoff, 1996).

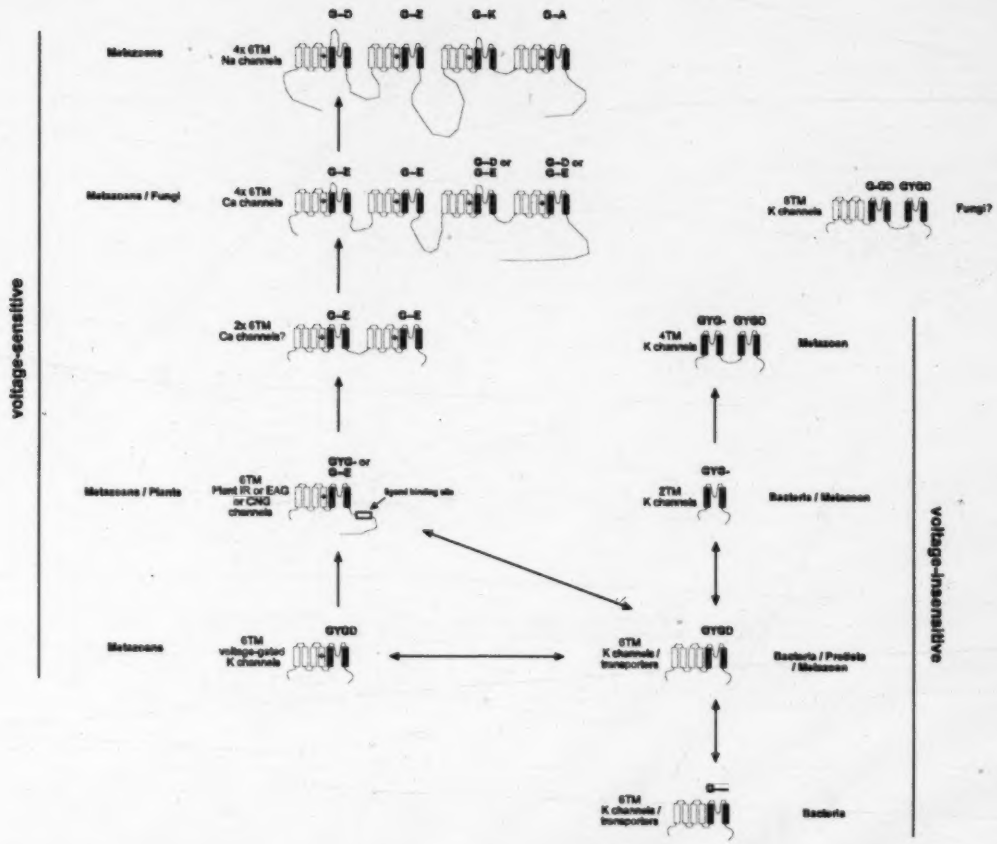

Figure 6-1. Parsimonious relationships among the structural classes of ion-selective channels with two, four and six transmembrane segments (2TM, 4TM, 6TM). Above each channel structure are the amino-acids that are expected to be important in ion selectivity. The earliest channels may not have been voltage-sensitive (voltage sensors indicated by "+"s) considering that K^+ channels from singled celled organisms (bacteria and Paramecium) and the only non-metazoan Ca^{2+} channel found in two yeast species do not have a well conserved pattern of charges in 4th segments. Both the 2TM and 6TM structural classes exist in bacterial species, so either structural class may be more ancient. CNG and closely-related EAG and plant inward rectifying channels are shown intermediate between K^+ and Ca^{2+} channels, because of the overall structural similarity with K^+ channels and the Ca^{2+}-like pore of CNG channels. Na^+ channels differ from K^+ and Ca^{2+} in being an almost exclusive property of metazoans. Na^+ channels are likely derivatives of a Ca^{2+} channel resembling a T-Type channel with four domains.

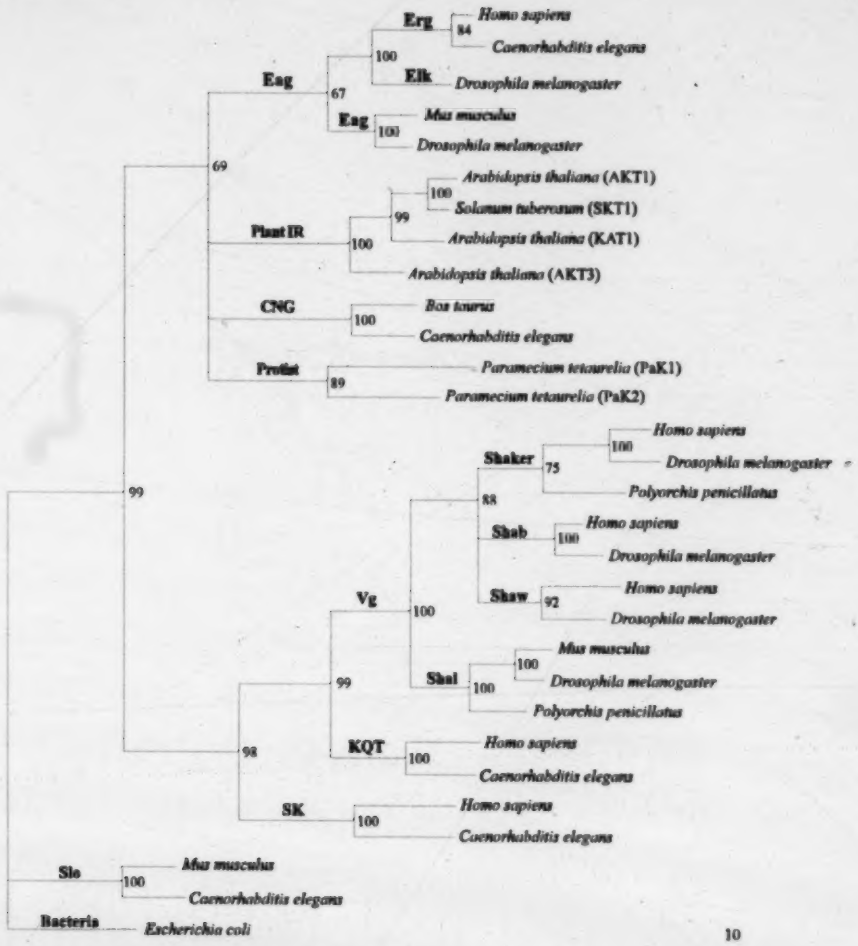

Figure 6-2. Phylogenetic tree of representative invertebrate and vertebrate voltage-gated K^+ channels generated using maximum parsimony. Bootstrap values for each branch point are shown. The tree contains the following genes, (with their common names, accession numbers): **Bacteria**: (*Escherichia coli*, L12044); **Slo**: *Caenorhabditis elegans* (nematode, Z68104), *Mus musculus* (mouse, U09383); **SK**: *Caenorhabditis elegans* (nematode, U39744), *Homo sapiens* (human, U69883); **KQT**: *Caenorhabditis elegans* (nematode, U41556), *Homo sapiens* (human, U40990); **Shal**: *Polyorchis penicillatus* (hydrozoan jellyfish, U78642), *Drosophila melanogaster* (fruit fly, M32660), *Mus musculus* (mouse, M64226); **Shaw**: *Drosophila melanogaster* (fruit fly, M32661), *Homo sapiens* (human, AF055989); **Shab**: *Drosophila melanogaster* (fruit fly, M32659), *Homo sapiens* (human, AF026005); **Shaker**: *Polyorchis penicillatus* (hydrozoan jellyfish, U32923), *Drosophila melanogaster* (fruit fly, M17211), *Homo sapiens* (human,L02752); **Protist**: ciliate *Paramecium tetaurelia* (ciliate: Pak1, U19907; Pak2, U19908); **CNG**: *Caenorhabditis elegans* (nematode, Z19157), *Bos taurus* (cow, Q00194); **Plant IR**: *Arabidopsis thaliana* (thale-cress: AKT1, U06745; KAT1, M86990; AKT3, U44745), *Solanum tuberosum* potato (SKT1, X86021); **Eag**: *Drosophila melanogaster* (fruit fly, Q02280), *Mus musculus* (mouse, U04294); **Elk**: *Drosophila melanogaster* (fruit fly, U04246); **Erg**: *Caenorhabditis elegans* (nematode, Z35596), *Homo sapiens* (human, U04270).

Table 6-1. Charged Amino Acids in 4th Segments of Representative Ion-Selective Channels

Accession #	Species Name	Channel Description*		Domain #	Amino Acid Sequence of S4 Segments
L12044	*Escherichia coli*	6TM K+ channel	kch		R F S I G F C I F T L V F L L I L L I L R R
U19907	*Paramecium tetraurelia*	6TM K+ channel	PaK1		S A A N Y L M L I R M T R V R R T M I V I E
U19908			PaK2		F D L Q Y L D L V I I L R V F Q I T R F S R
U32922	*Polyorchis penicillatus*	6TM OR Vg K+ channel (*Shaker*)	jShak1		S F S M L R V I R V L R V F R L S R H S R G
U32923	*Polyorchis penicillatus*		jShak2		V L R V V R V I R V I R I F R L T R H S R G
Z68104	*Caenorhabditis elegans*	6TM OR Slo K^+ channel	nSlo		A I Y L Q R N W L G F R F L R A L R L M T V
Z68104	*Caenorhabditis elegans*		nSlo2		V P V F L N C W L A R G A L Q A M M N D L N
U41556	*Caenorhabditis elegans*	6TM OR KQT K^+ channel	nKQT1		A I R G L R F F Q I L R M L R M L R I D R R
U39744	*Caenorhabditis elegans*	6TM OR SK K^+ channel	nSK1		L C R S Y L F A R F M V L H S R Q F Q D A S
X86021	*Solanum tuberosum*	6TM IR K+ channel	SKT1		L F N M L R L W R L R R V S S L F A R L E R
M86990	*Arabidopsis thaliana*	6TM IR K+ channel	KAT1		L L G L L R F W R L R R V K H L F T R L E R
Z35596	*Caenorhabditis elegans*	6TM OR Eag K^+ channel	nerg		L I G L L R T A R L L R L V R V A R R L D R
Z19157	*Caenorhabditis elegans*	6TM OR CNG K^+ channel	nCNG1		I L R L N R L I R Y K R V R N C L E A R T E
X87941	*Saccharomyces cerevisiaeae*	4x6TM Vg Ca^{2+} channel	X87941	1	G I R I F R P L A I L R I L R L V N V D T G
				2	W L S I F H I S R F Y R V I I S F N L T R R
				3	L S R I F R G L T A L R A L R A L R C L T I
				4	I R G F F L L V I F L F I I P Q N D T L T E
U55776	*Drosophila melanogaster*	4x6TM Vg Ca^{2+} channel (non-L type)	Dmca1	1	D L R T L R A I R V L R P L R L V S G I P S
				2	V L R A L R L L R I F R V T R Y W S S L R N
				3	T I R S L R V L R V L R V L R P L R T I R R
				4	F L R L F R A A R L I R L L R Q G Y T I R I
U93075	*Cyanea capillata*	4x6TM Vg Ca^{2+} channel (L type)	CyCa1	1	E V R A L R A F R V L R P L R L V S G V P S
				2	V L R C V R L L R I F R V T R Y W E S L S N
				3	L V R I L R V L R V L R P L R A I N R A R G
				4	F F R L F R A L R L V R L L S Q G D G I R T
L04569	*Homo sapiens*	4x6TM Vg Ca^{2+} channel (T type)	α1G	1	A V R T V R V L R P L R A I N R V P S M R I
				2	Q L R T F R L M R V L R L V R F L P A L Q R
				3	S L R V L R L L R T L R P L R V I S R A R G
				4	E I R I M R V L R I A R V L R L L R M A V G
M81758	*Homo sapiens*	4X6TM Vg Na^+ channel	SCN4A	1	A L R T F R V L R A L R T I T S V I P G L R T
				2	V L R S F R L L R V F R L A R S W P T L N L
				3	A I R S L R T L R A L R P L R A L S R F E G
				4	L F R V I R L A R I G R V L R L I R G A R G
M32078	*Polyorchis penicillatus*	4x6TM Vg Na^+ channel	PpSCN1	1	G I R T F R V L R A L R T I S T V K G L R A
				2	V L R S F R L L R V F R L A R S W P T L N M
				3	G L R A L R S L R G L R P L R A I S R F E G
				4	L F R V V R V F R L G R L L R F F E G A R G
M91556	*Homo sapiens*	4x6TM Vg Na^+ channel	SCN6A	1	T L Q T A R T L R I L R I I P L N Q G L R S
				2	L L R L F R M L R L F R L G R Y W P T F Q L
				3	E L R P L I S M R F L R P L R V L S Q F E R
				4	L V Q L I L L D T I I H M L R L G R G P R V

*TM = transmembrane segment(s), IR = inward rectifier, OR = outward rectifier, Vg = Voltage-Gated

Table 6-2. Amino Acid Sequences of Putative Pore-Forming Loops in Representative Ion-Selective Channels

Accession #	Species Name	Channel Description*		Domain #	Amino Acid Sequence of P-Loop
U60209	*Xenopus laevis*	2 x 6TM CFTR Cl^- channel	CFTR		A Y V R Y F N S S A F F F S F V V F L
X60433	*Torpedo californica*	12 TM Vg Cl^- channel	ClC-0		T H A V S T A V I C F E L T Q I S H V L
X77933	*Rattus norvegicus*	Epithelial Na^+ channel (γ subunit)	rENaC		S P A N S I E M L L S N F G Q L G L W M
X02870	*Escherichia coli*	10 TM high affinity K^+ uptake transporter	KdpA	1	A S Q E A I K M L G T N G G F N A N S
X56783	*Escherichia coli*	10 TM low affinity K^+ uptake transporter	TrkGA	1	L N L T F I D A L F E G V S I T T T G A
U08371	*Escherichia coli*	2 TM mechanosensitive channel	mscL		R D A Q G D I P A V V M H Y V F I Q N V
Z37989	*Streptomyces lividans*	2TM K+ channel / transporter	skc1		P R A L W W S V E T A T T L Y P
L12044	*Escherichia coli*	6TM K+ channel / transporter	Kch		M T A F Y F S I E T M S T I V P
X05087	*Bacillus stearothermophilus*	6TM K+ channel / transporter	LctB		E D S L Y L S G M T L L S V T P
U19807	*Paramecium tetraurelia*	6TM K+ channel / transporter	PaK1		F H S L Y W I T I T S M T I V P
U28005	*Saccharomyces cerevisiae*	8TM OR Two-Pore K^+ channel	TOK1	1	G N A L Y F C T V S L L T L I L P
				2	F H C I Y F C F L C L L T Y A P
U40947	*Caenorhabditis elegans*	2TM IR K^+ channel	nirk		Y S S F L F A V E T H H T H R Y I
L12018	*Caenorhabditis elegans*	4TM OR Two-Pore K^+ channel	F22B7a	1	S S S I F F A V T V V T T N P V P
				2	F T S F Y W S F I T M T T L M P
U03632	*Homo sapiens*	4TM OR Two-Pore K^+ channel	TWIK1	1	T S A L F F A S T V L S T T H T V P
				2	L E S F Y F C F I S L S T L Y V P
U32922	*Polyorchis penicillatus*	6TM OR Vg K+ channel (*Shaker*)	jShak1		P S S F W W A I V T M T T M H P
U41566	*Caenorhabditis elegans*	6TM OR KQT K^+ channel	nKQT1		A D A L W W G V I T L S T K T P
Z68104	*Caenorhabditis elegans*	6TM OR Slo K^+ channel (maxi-K)	nSlo		A D S V Y F V L V T M S T I Y C
U39744	*Caenorhabditis elegans*	6TM OR SK K^+ channel	nSK1		S N S L W F I A I T F M L I V P
X86021	*Solanum tuberosum*	6TM IR K+ channel	SKT1		I T S L Y W S I V T L T T T L H A
M86890	*Arabidopsis thaliana*	6TM IR K+ channel	KAT1		V T A L Y W S I T T L T T T F H A
Z35636	*Caenorhabditis elegans*	6TM OR Eag K^+ channel	nerg		V T S L Y F T L S T I T S N V S A
Z19157	*Caenorhabditis elegans*	6TM OR CNG K^+ channel	nCNG1		V Y S F Y W S T L I L T T T V P S
X87941	*Saccharomyces cerevisiae*	4x6TM Vg Ca^{2+} channel	X87941	1	N I V N S M E L V F V I M S A - - N T F T
				2	S L P N S F L S L F I I G S T - - I N W T
				3	S F A S A F S S L Y Q I I S L - - I G W V
				4	T V I K S M I V L F R C S F G - - E G W N
U55776	*Drosophila melanogaster*	4x6TM Vg Ca^{2+} channel (non-L)	Dmca1	1	N I G F A M L T V F Q C I T M - - I G W T
				2	T F P I A L L T V F Q I L T G - - E D W N
				3	N V A A A M L T L F A V Q T G - - I G W P
				4	S F I H G V M L L F R C A T G - - E A W P
U93075	*Cyanea capillata*	4x6TM Vg Ca^{2+} channel (L)	CyCa1	1	N V A L A A L T V F Q C T T L - - I G W T
				2	S F W R S L I T V F Q I L T G - - I W N A
				3	N V F Q A Y L S L F V V M T F - - E G W P
				4	T F P Q A L I V L F R S A T G - - E N W Q
L04569	*Caenorhabditis elegans*	4x6TM Vg Ca^{2+} channel (T-Type)	α_{1G}	1	N I G F A W V A I F L V I S L I G W T
				2	T L L W A I I T V F Q I L T Q E D W N
				3	N L G Q A L M S L F V L S S K E G W V
				4	N F G M A F L T L F R I A T G - - E N W N
M81758	*Homo sapiens*	4X6TM Vg Na^+ channel (TTX-sensitive)	SCN4A	1	T F S W A F L A L F R L M T Q - - I Y W E
				2	D F F H S F L I V F R I L C G - - I W I E
				3	N V G L G Y L S L L Q V A T F - - K G W M
				4	T F G N S I I C L F E I T T S - - A G W D
M77235	*Homo sapiens*	4X6TM Vg Na^+ channel (TTX-resistant)	SCN5A	1	S F A W A F L A L F R L M T Q - - I G W E
				2	D F F H S F L I V F R I L C G - - I W I E
				3	N V G A G Y L A L L Q V A T F - - K G W M
				4	T F A N S M L C L F Q I T T S - - A G W D
AF041851	*Aiptasia pallida*	4X6TM Vg Na^+ channel	AnemNa1	1	N F G W A V I T A F Q L V T L I Y W E
				2	D F W H S F M M I F R V L C G K W I E
				3	N S L N G F M A L F Q V A T F I G W I
				4	T F G N S M L L L F R L S T S A G W N
L15445	*Cyanea capillata*	4X6TM Vg Na^+ channel	CYNA1	1	N F L L A M L T S L Q V C T L I Y W E
				2	N F G N S F M M I F R I L C G K W I E
				3	N S I S G F L A L F Q T A T L I G W F
				4	T F G S S M C L L F R I S T A A G W N
AF047380	*Polyorchis penicillatus*	4X6TM Vg Na^+ channel	PpSCN1	1	N F L S A M L T S L Q V C T L I Y W E
				2	N F G N S F M M I F R I L C G K W I E
				3	N S I N G F L A L F Q T A T L E G W F
				4	T F G R S M C L L F R I S T A A G W N

*TM = transmembrane segment(s), IR = inward rectifier, OR = outward-rectifier, Vg = Voltage-Gated

The cyclic nucleotide-gated (CNG) channels seem to be intermediate between K^+ and Ca^{2+} channels with a pore that resembles Ca^{2+} channels and an overall structural similarity to K^+ channels (Yau and Tsung-Yu, 1995). In particular, CNG channels belong to a family including closely related plant inward rectifying and Eag channels that are similar in sequence (fig.6-2) and share a cyclic nucleotide binding site (fig. 6-1) (Yau and Tsung-Yu, 1995).

The four domains of Na^+ and Ca^{2+} channels have a weak structural similarity to the single domain of K^+ channels and thus must have diverged substantially if their origins were from K^+ channels (fig. 6-3). The domains of cation channels resemble the voltage-gated, SK or KQT subfamily of K^+ channels more closely than the CNG subfamily, as might be expected from the apparent likeness of the CNG subfamily pores to Ca^{2+} channel pores (fig 6-3).

B. Emergence of separate roles played by Ca^{2+} and Na^+ ions

The concentration of Ca^{2+} and Na^+ ions are kept low in cells, so that open channels have a steep electrical and chemical gradient driving these cations into cells (Guyton, 1997). Unlike these cations, potassium is the predominant cation in cells and is tightly-linked to maintaining the ionic and electrical state of cells (Guyton, 1997).

Ca^{2+} and Na^+ ions differ in their roles as inward currents. Ca^{2+} ions are a major link between depolarization and cell activity, being the on / off switch for cellular

processes like contraction, secretion, gene regulation and metabolism (Hille, 1992b). Likely because of this intimate connection with cytoplasmic activity, cells are extremely sensitive to Ca^{2+} ions, with a normal intracellular concentration of Ca^{2+} in the 20 to 300 nM range, but never exceeding 1μM in an excitable cell (Hille, 1992b). Ca^{2+} ions are tightly regulated by Ca^{2+} transport and sequestering proteins present in the plasma membrane and in membrane-bounded organelles, like the mitochondria and the sarcoplasmic reticulum in muscle (Hille, 1992b).

On the other hand, Na^+ ions are more tolerated in cells with concentrations that may reach up to 5mM (Hille, 1992b). It is the absence of a link to cell processes that allows Na^+ ions to exploit the steep electrochemical gradient to a greater extent than Ca^{2+} ions. One role that Na^+ ions play is to drive the uphill transport of other ions (antiport with H^+ or Ca^{2+}) and solutes (eg. cotransport of glucose, amino-acids) via the electrochemical gradient in non-excitable cells (Guyton, 1997).

In excitable cells, the major role for Na^+ ions is to create a rapid voltage change across membranes by large, rapid fluxes of ionic current through voltage-gated Na^+ channels. Channel densities for Na^+ channels, for example, may exceed 2000 channels / μm^2 in nodes of Ranvier to take advantage of the steep electrochemical gradient for Na^+ ions (Chiu, 1980; Conti *et al.*, 1976). The steepness of the upstroke created by the Na^+ current is one of the parameters responsible for the fast propagation of action potential waves.

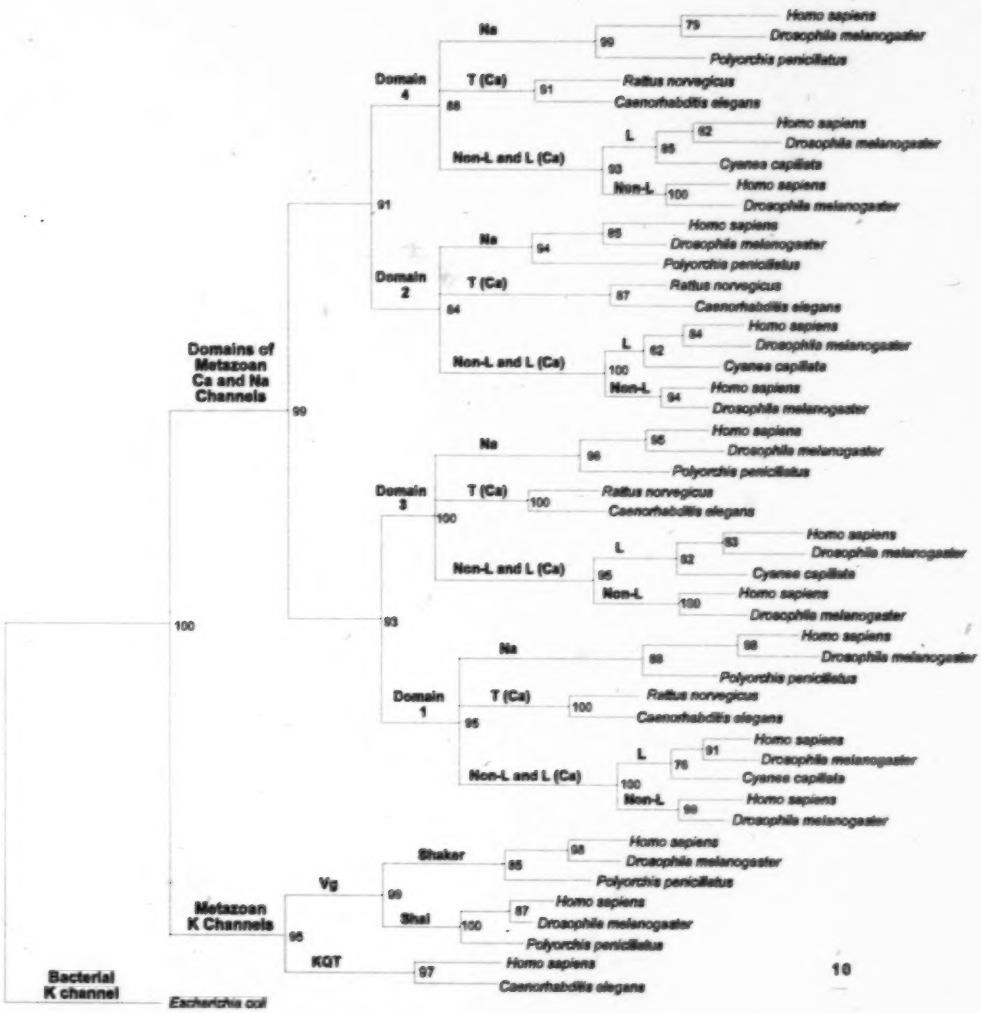

Figure 6-3. Phylogenetic tree of the four domains of representative Ca^{2+} and Na^{+} channels, rooted with K^{+} channels and generated using maximum parsimony. Bootstrap values for each branch point are shown. The tree contains the following genes, (with their common names, accession numbers): **K^{+} channels**: Bacteria: (*Escherichia coli*, L12044); KQT: *Caenorhabditis elegans* (nematode, U41556), *Homo sapiens* (human, U40990); Shal: *Polyorchis penicillatus* (hydrozoan jellyfish, U78642), *Drosophila melanogaster* (fruit fly, M32660), *Mus musculus* (mouse, M64226); Shaker: *Polyorchis penicillatus* (hydrozoan jellyfish, U32923), *Drosophila melanogaster* (fruit fly, M17211), *Homo sapiens* (human,L02752); **T-type Ca^{2+} channels**: *Caenorhabditis elegans* (nematode, U37548), *Rattus norvegicus* (rat, AF027984); **non-L type Ca^{2+} channels**: *Drosophila melanogaster* (fruit fly, U55776), *Homo sapiens* (human, X99897); **L-type Ca^{2+} channels**: *Cyanea capillata* (scyphozoan jellyfish, U93075); *Drosophila melanogaster* (fruit fly, U00690); *Homo sapiens*, (human, L29529); **Na^{+} channels**: *Polyorchis penicillatus* (hydrozoan jellyfish, AF047380); *Drosophila melanogaster* (fruit fly, M32078), *Homo sapiens* (human, M81758).

Ca^{2+} ions can carry the action potential without Na^{+}-selective channels, for example in the pacemaker of the mammalian heart where the pacemaker signal is relatively slow, or the enteric nervous system, where slow Ca^{2+} oscillations and contractions of the gut are mediated by external Ca^{2+} ions through Ca^{2+} channels (Guyton, 1997). An intermediate system uses both Ca^{2+} and Na^{+} channels in the same waveform, for example in the ventricular action potential of the mammalian heart. A very steep waveform of the action potential is required to generate a rapidly transmitting signal that reaches all parts of the ventricle quickly, so contractions are synchronized. Ca^{2+} is carried in the plateau of the waveform, and the amount of extracellular Ca^{2+} flowing through Ca^{2+} channels corresponds to the strength of the ventricular contraction (Guyton, 1997). In its most differentiated state, the cation current is carried almost exclusively by Na^{+} ions, propagating rapidly along long, thin axons of nervous tissue that terminate, for example at the motor end plate (Guyton, 1997). Unlike cardiac muscle, skeletal muscle contracts in the absence of extracellular Ca^{2+} ions, thus providing an effector that is more separated from the afferent signal carried by the nervous system.

C. Evolution of Na^+ from Ca^{2+} channels

Na^+ channels likely evolved late from Ca^{2+} channels resembling T-type channels by small structural changes, and not independently from K^+ channels (fig. 5-2). Na^+ channels also resemble T-Type channels kinetically. They are both transient, inward currents that are rapidly activating and inactivating.

The kinship of the channel domains can be traced in Na^+ and Ca^{2+} channels, suggesting that there was a period of selection following each duplication round, allowing each domain to alter its structure. In the topology of the most parsimonious trees of Na^+ and Ca^{2+} channel domains, domains I and III as well as domains II and IV of both channel types appear to be more similar to each other than the domains within each channel type separately (fig. 6-3) (Strong *et al.*, 1993). This suggests that the Na^+ and Ca^{2+} channels both evolved from a primordial single chain, four domain channel that had already undergone two rounds of duplication.

The close kinship between Na^+ and Ca^{2+} channels is evident in the similarity of their channel pores. Most Na^+ channels have two negatively charged residues, and a positively charged (K), and neutral (A) residues in the position where the four negatively-charged residues reside in pore of Ca^{2+} channels (Table 6-2). Modification of the non-negatively charged "K" and "A" residues in Na^+ channels to negative charges (Heinemann *et al.*, 1992b) or phosphorylation of protein kinase A sites (Santana *et al.*, 1998) can produce Ca^{2+} rather than Na^+ selectivity. Unlike the highly selective 1000:1

fold selectivity of the Ca^{2+} channel for Ca^{2+} ions over their nearest competitor, Na^+ channels are only 10:1 times more selective for Na^+ ions over Ca^{2+} ions (Hille, 1992b). The lower affinity of Na^+ ions for Na^+ channels is not surprising, considering that they lack the double charge of Ca^{2+} ions and their channels lack the symmetrical, high affinity pocket of Ca^{2+} channels. Thus, the transition from Ca^{2+} to Na^+ channels appears to be a small step, only a matter of a couple mutations disrupting the high-affinity "EGTA"-like binding sites of Ca^{2+} channels.

D. Cnidaria and the evolution of Na^+ channels

The development of Na^+ channels, as specialized proteins for producing rapid, transcellular signals with high frequency signalling capability, is a cornerstone of the evolution of metazoan nervous systems. Nervous systems bearing Na^+ channels likely evolved out of necessity in multi-cellular organisms, which have tissues, as well as cells, acting as semi-autonomous, but interacting units. These require a high level of coordination and feedback.

Cnidarians are the simplest extant eumetazoans with a discrete nervous system and have rapid, transient Na^+ currents. They are diploblastic animals and are considered to have split from the main lineage early in metazoan evolution (Morris, 1993; Wainright *et al.*, 1993). Cnidarians may provide clues to possible primitive, features of ancestral

Na^+ channels and thus, cnidarians are suitable organisms for providing possible clues to ancestral nervous systems. Na^+ currents in the hydromedusa, *Polyorchis penicillatus* are unusually insensitive to classical Na^+ channel drugs, like tetrodotoxin, but do not have a pharmacological profile that is altogether "Ca^{2+}-like" either. Na^+ currents in swim motor neurons (SMNs) and B neurons have differing electrophysiological features that may be shaped respectively, by the convergent "cardiac-like" or "neuronal-like" roles that they may play. A Na^+ channel gene isolated from *P. penicillatus* has major structural features of typical Na^+ channels, especially in areas responsible for ion selectivity, voltage-sensing, fast-inactivation and glycosylation. Homology extends to position of introns and the different intron splicing mechanisms employed between *P. penicillatus* and mammalian Na^+ and Ca^{2+} channel genes. The similarity of features of Na^+ channels and currents in *P. penicillatus* suggest that the organization and fundamental composition of the genetic template for Na^+ channels has undergone few changes since it was laid down in the common ancestors of diploblasts and triploblasts, ~600 million to 1 billion years ago (Morris, 1993).

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APPENDIX A cDNA SEQUENCE OF PpSCN1[14]

```
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DEFINITION  Polyorchis penicillatus voltage-gated sodium channel alpha subunit
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ACCESSION   AF047380
NID         g3005563
KEYWORDS    .
SOURCE      Polyorchis penicillatus.
  ORGANISM  Polyorchis penicillatus
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            Polyorchidae; Polyorchis.
REFERENCE   1  (bases 1 to 5806)
  AUTHORS   Spafford,J.D., Spencer,A.N. and Gallin,W.J.
  TITLE     A putative voltage-gated sodium channel alpha subunit (PpSCN1) from
            the hydrozoan jellyfish, Polyorchis penicillatus: structural
            comparisons and evolutionary considerations
  JOURNAL   Biochem. Biophys. Res. Commun. 244, 772-780 (1998)
REFERENCE   2  (bases 1 to 5806)
  AUTHORS   Spafford,J.D., Spencer,A.N. and Gallin,W.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-FEB-1998) Department of Biological Sciences,
            University of Alberta, Biological Sciences Building, Edmonton,
            Alberta T6G 2E9, Canada
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[14] A version of this appendix has been published. Spafford, J.D., Spencer, A.N. and Gallin, W.J. (1998) GenBank Sequence Database, National Center for Biotechnology Information, National Library of Medicine Building 38A, Bethesda, MD 20894. *(Accessible by query at the following World Wide Web Site http://www2.ncbi.nlm.nih.gov/genbank/query_form.html)*

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APPENDIX B GENOMIC SEQUENCE OF PPSCN1[15]

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NID         g3005561
SOURCE      Polyorchis penicillatus.
  ORGANISM  Polyorchis penicillatus
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            Polyorchidae; Polyorchis.
REFERENCE   1  (bases 1 to 13909)
  AUTHORS   Spafford,J.D., Spencer,A.N. and Gallin,W.J.
  TITLE     Genomic organization of a voltage-gated sodium channel from a
            primitive metazoan (PpSCN1): evolutionary considerations

  TITLE     Direct Submission
  JOURNAL   Submitted (09-FEB-1998) Department of Biological Sciences,
            University of Alberta, Biological Sciences Building, Edmonton,
            Alberta T6G 2E9, Canada
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[15] A version of this appendix has been published. Spafford, J.D., Spencer, A.N. and Gallin, W.J. (1998) GenBank Sequence Database, National Center for Biotechnology Information, National Library of Medicine Building 38A, Bethesda, MD 20894. *(Accessible by query at the following World Wide Web Site http://www2.ncbi.nlm.nih.gov/genbank/query_form.html)*

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APPENDIX C LIST OF PUBLICATIONS

PAPERS IN REFEREED JOURNALS

1. Spafford, J.D., Spencer, A.N. and Gallin, W.J. Genomic organization of a voltage-gated Na^+ channel (PpSCN1) in a primitive metazoan: *Evolutionary* implications. (submitted 1998).

2. Grigoriev, N.G., Spafford, J.D. and Spencer, A.N. Positive feed-back of potassium currents by effluxing potassium: effects of levels of expression of a jellyfish *Shaker* channel (*jShak2*) in *Xenopus* oocytes (submitted 1998)

3. Grigoriev, N.G., Spafford, J.D. and Spencer, A.N. Residues in the jellyfish Shaker-like channel (*jShak2*) involved in modulation by external potassium (submitted 1998)

4. Grigoriev, N.G., Spafford, J.D. and Spencer, A.N. Modulation of jellyfish *Shaker* K^+ channels by external potassium ions. (submitted 1998).

5. Spafford, J.D., Spencer, A.N. and Gallin, W.J. A putative voltage-gated sodium channel α subunit (PpSCN1) from the hydrozoan jellyfish, *Polyorchis penicillatus*: structural comparisons and evolutionary considerations. Biochem. Biophys. Res. Comm. 244:3:772-780 (1998).

6. Grigoriev, N.G., Spafford, J.D., Gallin, W.J. and Spencer, A.N. Voltage-sensing in jellyfish *Shaker* K^+ channels J. Exp. Biol. 200:22:2919-2926 (1997).

7. Grigoriev, N.G., Spafford, J.D., Przysiezniak, J.P. and Spencer, A.N. A cardiac-like sodium current in motor neurons of jellyfish. J. Neurophys. 76:4:2240-49 (1996).

8. Spafford, J.D., Grigoriev, N.G. and Spencer, A.N. Pharmacological properties of voltage-gated Na^+ currents in motor neurons from a hydrozoan jellyfish Polyorchis penicillatus. J. Exp. Biol. 199:4:941-948. (1996).

ABSTRACTS

1. Spafford, J.D., Spencer, A.N. and Gallin, W.J. The genomic organization of a sodium channel gene in a hydrozoan jellyfish. Soc. Neurosci. Abstr. 23:(1):911 (1997).

2. Grigoriev, N.G., Spafford, J.D., Gallin, W.J. and Spencer, A.N. Voltage-sensing in a jellyfish *Shaker* K^+ channel (JSHAK1). Soc. Neurosci. Abstr. 22(2):1249 (1996).

3. Spafford, J.D., Grigoriev, N.G., Gallin, W.J. and Spencer, A.N. Structural and functional features of voltage-gated sodium channels in a hydrozoan jellyfish. Soc. Neurosci. Abstr. 22(1):57 (1996).

4. Grigoriev, N.G., Spafford, J.D., Gallin, W.J. and Spencer, A.N. Mutations in the S4 region of a jellyfish potassium channel. Soc. Neurosci. Abstr. 21(2):1327 (1995).

5. Spafford, J.D., Spencer, A.N. and Gallin, W.J. Cloning of a voltage-gated sodium channel from the hydrozoan jellyfish *Polyorchis penicillatus*. Biochem. Cell Biol. 71:Axx (1993).

GENBANK SUBMISSIONS

1. PpSCN1 gene, Accession # AF047379

2. PPSCN1 mRNA, Accession # AF047380